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13. ABSTRACT *(Maximum 200 Words)*

Oral antiviral drugs would be highly desirable for the prevention and treatment of smallpox biowarfare or bioterrorism casualties. During the first year of this grant, we synthesized 1-O-hexadecyloxypropyl-cidofovir (HDP-CDV), a novel ether lipid prodrug of cidofovir (CDV) and 20 related analogs. In cells infected with variola, monkeypox, cowpox or vaccinia viruses, HDP-CDV inhibited the replication of all test viruses with a 50% effective concentration (EC_{50}) between 0.008 and 1 µM while CDV showed EC_{50} values between 4.3 and 36 µM. HDP-CDV was 93% orally bioavailable in mice and provided plasma drug levels well above the *in vitro* EC_{50} for various poxviruses. In collaboration with USAMRIID investigators, oral HDP-CDV was given daily for 5 days to BALB/c mice lethally challenged with cowpox virus. HDP-CDV treated mice had 100% survival at 5 and 10 mg/kg/day and 90% at 20 mg/kg/day, while oral doses of CDV of 10 or 20 mg/kg/day were ineffective. Our data suggest that further evaluation of HDP-CDV is indicated for potential use in the prevention or treatment of smallpox. We also synthesized and tested over 20 analogs of CDV and cyclic CDV which are being evaluated *in vitro* and *in vivo*. Finally, we also identified several new antiviral phosphonate derivatives which are not based on cidofovir. The new compounds are highly active against smallpox, vaccinia and cowpox. These backup compounds are about 1-2 years behind HDP-CDV in development and will be evaluated further during the 02 year of this project.

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INTRODUCTION

Smallpox (variola major) is estimated to have killed a billion persons over the past 200 years. Vaccination eliminated the disease from the earth in the late 1970s (WHO) but stocks of smallpox have been retained by the US and Russia at safe locations. However, rogue states such as Iraq, North Korea, Iran or terrorist groups may also have stocks of smallpox which could be used in biowarfare or bioterrorism. In the late 80s, Russia produced tons of variola, India-1, for use in long range ICBMs (Alibek) and it is uncertain what has become of those stocks and the thousands of persons trained to produce the variola virus.

The US population has not been vaccinated since the late 70s and is therefore highly susceptible to infection. Although sufficient vaccine has been identified for use in a national emergency, vaccine reactions may be severe in persons with weak immune systems (AIDS, Cancer or Organ/bone marrow transplants) or in patients with skin conditions like eczema and atopic dermatitis. For these reasons, it would be useful to have a safe and effective oral agent which could be used to prevent or treat variola infection for use in persons in whom vaccination would be to dangerous.

Furthermore, interleukin-4 (IL4+) positive strains of poxvirus have been reported by Australian researchers which may be able to circumvent vaccine immunity. This genetically engineered approach, if applied to variola, could produce a smallpox virus which would be effective in causing illness and death, even in vaccinated persons. Therefore, an effective antiviral countermeasure would be highly useful, particularly one which works orally and could be self administered easily.

In the first year of this project, we have synthesized over 20 highly active and selective orally active derivatives of cidofovir and cyclic cidofovir and assessed their in vitro activity and cytotoxicity. Some of the more promising agents have been successfully tested in vivo in lethal models of poxvirus disease (HDP-CDV; ODE-CDV, OLP-CDV and OLE-CDV). We have also identified two other classes of highly active poxvirus drugs which are NOT based on cidofovir. These new compounds are 8.8 to 73 times more active than HDP-CDV *in vitro* against vaccinia virus and 5 to 32 times more active than HDP-CDV versus cowpox virus.

In the 02 year of this project, we will further evaluate analogs of these new agents, while providing larger amounts of the leading two cidofovir analogs to USAMRIID colleagues for *in vivo* testing in small animals and in primates.

BODY:

Specific Goal #1: To Carry out Structure-Activity Assessments of the *in vitro* Activity and Selectivity of Ether Lipid Prodrugs of Cidofovir Designed for Enhanced Activity Against Smallpox and Other Orthopox Viruses.

1.1 Structure-Activity assessments of CDV and cyclic CDV versus their ether lipid analogs versus vaccinia and cowpox *in vitro.*

Brad Wan, James Beadle and Stephanie Ciesla of our laboratory, synthesized a series of alkoxyalkanol esters of cyclic CDV and CDV. The compound structures, abbreviations and Antiviral Research Branch numbers (ARB numbers) are shown below.

Table 1.1.1 Derivatives of CDV and cyclic CDV synthesized and submitted to the Antiviral Research Branch for antiviral testing:

ARB#	Compound Name	Abbreviation
CDV Analogs		
00-394	1-O-hexadecylpropanediol-CDV	HDP-CDV
00-480	1-O-octadecylethanediol-CDV	ODE-CDV
01-491	1-O-octadecylpropanediol-CDV	ODP-CDV
02-130	1-O-oleyloxypropyl-cidofovir	OLP-CDV
02-131	Hexadecyl-cidofovir	HD-CDV
02-143	1-O-octadecylglycero-3-CDV	ODG-CDV
02-360	1-O-oleyloxyethyl-CDV	OLE-CDV
02-487	1-O-octadecylglycero-CDV	ODG-CDV
02-489	1-O-octadecyl-2-O-benzyl-*sn*-glycero-3-CDV	ODGB-CDV
02-554	1-O-dodecyloxypropyl-CDV	DDP-CDV
02-555	1-O-octyloxypropyl-CDV	OP-CDV
Cyclic CDV Analogs		
00-393	1-O-hexadecylpropanediol-cyclicCDV	HDP-cCDV
00-479	1-O-octadecylethanediol-cyclicCDV	ODE-cCDV
01-489	Hexadecyl-cyclicCDV	HD-cCDV
01-490	1-O-octadecylpropanediol-cyclicCDV	ODP-cCDV
02-490	1-O-octadecyloxypropyl-3-cyclic CDV	ODP-cCDV
02-491	1-O-octadecyl-2-benzyl-glycero-3-cyclic CDV	ODGB-cCDV
02-492	1-O-oleyloxypropyl-cyclic CDV	OLP-cCDV
02-493	1-O-oleyloxyethyl-cyclic CDV	OLE-cCDV

These compounds were tested for efficacy (effective dose 50%, EC_{50}) against cowpox and vaccinia virus in infected HFF cells (Table 1.1.2). Cytotoxicity (toxic dose 50%, TC_{50}) in HFF cells was also determined. Selectivity is the TC_{50}/EC_{50}. Portions of this table have been published (Kern et al, Antimicrob Agents Chemother 46:991-5, 2002) and this publication is included in the appendix. The details of the chemistry are presented in the publication and have not been included in the Body of this report.

Table 1.1.2 Effect of CDV and cyclic CDV analogs on antiviral activity

			Vaccinia				Cowpox	
	Compound	EC_{50} (μM)[a]	TC_{50} (μM)	Selectivity TC_{50}/EC_{50}		EC_{50} (μM)[a]	TC_{50} (μM)	Selectivity TC_{50}/EC_{50}
CDV	1a	31 ± 3.5	> 317	> 10		37.9 ± 8.1	> 317	> 8
HDP	2a	0.7 ± 0.45	31	39		0.56 ± 0.28	31	52
ODP	3a	1.2 ± 0.5	28.7	23.9		1.9 ± 0.6	28.7	15
ODE	4a	0.21 ± 0.07	14.3	72		0.23 ± 0.2	14.3	62
OLP	5a	0.3	69.4	231		0.33	69.4	210
OLE	6a	0.059 ± 0.02	14.3	242		0.071 ± 0.02	14.3	201
ODGB	7a	0.44 ± 0.11	69.4	158		0.3 ± 0.01	69.4	231
DDP	10a							
HD	8a	3	90.1	30		7.4	90.1	12.2
OP	9a	> 20	> 100	> 5		> 20	> 100	> 5
cCDV	1b	40.4 ± 8.1	> 331	> 8		45.3 ± 11.5	> 331	> 7
HDP	2b	3.8	> 100	> 26		2.1	> 100	> 48
ODP	3b	12 ± 1.1	74.7	6.2		14 ± 4.2	74.7	5.3
ODE	4b	1.1	47.8	43.4		0.5	47.8	95.6
OLP	5b	0.45 ± 0.13	45.9	102		0.38 ± 0.08	45.9	121
OLE	6b	0.42 ± 0.02	26.4	63		0.33 ± 0.06	26.4	80
ODGB	7b	8.6 ± 1.3	> 100	> 12		5.8 ± 3.7	> 100	> 17
HD	8b	> 20	68.5	> 3.4		> 20	68.5	> 3.4
OP	9b	> 20	> 100	> 5		> 20	> 100	> 5

Testing in Human Foreskin Fibroblasts (HFF). EC_{50} values done by plaque reduction assays. Vaccinia virus was Copenhagen Strain. Cowpox virus was Brighton Strain.
[a]Values are the mean of two or more assays.

Discussion of Table: It is noteworthy that all of the esters synthesized are more active than unmodified CDV with the exception of the short chain 8 carbon compound 1-O-octyloxypropyl-CDV (OP-CDV). The CDV series was more active and selective than the cyclic CDV series. The most active and selective compounds were: HDP-CDV, ODE-CDV, OLP-CDV, OLE-CDV and ODGB-CDV. Finally, these novel CDV analogs are also highly active against HCMV, HSV-1 and HSV-2 (Beadle et al, Antimicrobial Agents & Chemotherapy,46:2381-6, 2002).

1.2 Activity of CDV and Cyclic CDV analogs against smallpox and monkeypox *in vitro*.

In collaboration with Dr. John Huggins of USAMRIID, we submitted the analogs of CDV and cyclic CDV for testing in vitro Vero 76 cells or MK-2 cells infected with variola major, Bangladesh, or monkeypox, Zaire 79. Results are expressed as the 50% micromolar effective concentration. As shown in Table 1.2.1. below, CDV is generally more active than cyclic CDV against cells infected with smallpox or monkeypox *in vitro*. HDP-CDV and ODE-CDV are 65 to 1020 times more active than CDV. Similar trends are seen for HDP-cCDV and ODE-cCDV versus cCDV. In general, HDP-CDV and ODE-CDV are 7 to 180 times more active than the corresponding HDP-cCDV and ODE-cCDV analogs.

Table. 1.2.1 *In vitro* effect of ether lipid analogs of cidofovir or cyclic cidofovir on variola or monkeypox virus replication*

	EC_{50}, micromolar			
	Smallpox Bangladesh		Monkeypox Zaire 79	
Drug	Vero76	MK2	Vero76	MK2
CDV	27.3	10.2	4.6	4.3
HDP-CDV	0.10	0.04	0.07	0.013
ODE-CDV	0.03	0.01	0.006	0.006
cCDV	45.8	12.3	63.6	4.3
HDP-cCDV	0.9	0.6	1.8	0.09
ODE-cCDV	0.9	0.1	1.1	0.07

Testing in Vero 76 and MK-2 cells done by neutral red reduction assay.
* Data of John Huggins and coworkers, USAMRIID.

We have not yet submitted all of the new analogs of CDV (Table 1.1.1) for testing against variola but will do so during the 02 year of this project. We are particularly interested in OLP-CDV and OLE-CDV, which are very active and selective against vaccinia and cowpox (Table 1.1.2)

1.3 Activity of HDP-CDV and related compounds against Mousepox and IL-4 Modifed Mousepox (Ectromelia):

Previous experiments by Australian researchers indicated that the IL-4+ strain could circumvent immunization in mice, raising fears that this maneuver, if applied to smallpox, could circumvent vaccination. It is therefore of substantial interest to explore the effect of HDP-CDV and related compounds on the IL-4+ strains of ectromelia. Dr. R. Mark Buller of St. Louis University reported the following data in Ectromelia (mousepox) and IL4 positive Ectromelia using HDP-CDV and other analogs.

Table 1.3.1. Antiviral Activity of cidofovir and alkoxyalkyl esters versus Ectromelia, wild type, and Ectromelia IL4-modified Strains

50% inhibitory conc[1] □M		
COMPOUND	**ECTV-wild type**	**ECTV-7.5E-mIL-4+**
CDV	10.00	12.00
HDP-CDV	0.40	0.18
ODE-CDV	0.23	0.16
OLE-CDV	0.20	0.16

[1]Plaque reduction assay in CV-1 cells

Using a CV-1 cell plaque reduction assay with either ECTV-wt or an ECTV-IL-4+ recombinant virus as the indicator virus, Dr. Buller observed that all 3 tested alkoxyalkyl esters of cidovofir showed enhanced antiviral potency as compared to unmodified cidovovir (Table 1.3.1).

1.4. New, Highly Active Anti-Poxvirus Backup Compounds for Orthopoxvirus Synthesized and Evaluated *in vitro*:

Recently, Dr. Brad Wan, Dr. Nadejda Valiaeva and Stephanie Ciesla, of our laboratory, synthesized several ether lipid analogs of other phosphonate nucleotides and found them to be highly active against herpesviruses. In collaboration with Earl Kern and Kathy Keith of the University of Alabama at Birmingham, these compounds were tested in vitro against vaccinia and cowpox viruses versus HDP-CDV, our present lead compound. Preliminary results for 3 replicates are given as □M concentration to give 50% reduction in viral plaques are given in below. HDP-PMEG and HDP-(S)HPMPA were also tested against smallpox and monkeypox at USAMRIID and were found to be highly active with EC50 values less than 0.05. The data for variola (Bangladesh, BSH) or monkeypox are either one or two replicates as indicated.

Table 1.4.1. Antiviral Activity of Novel Ether Lipid Backup Compounds against Vaccinia, Cowpox, Variola (Bangladesh) or Monkeypox Viruses *in vitro

	50% inhibitory conc, μM[1]			
Compound	Vaccinia	Cowpox	Variola/BSH	Monkeypox
HDP-CDV	0.8	0.600	1.1; 0.1	3.8; 0.07
HDP-PMEG	0.091	0.120	<0.05	<0.05
HDP-(S)HPMPA	0.011	0.019	<0.05	<0.05

** Data of Dr. Earl Kern (VAC,CPX) or John Huggins & coworkers (V/BSH,MPX)*
[1] Plaque reduction assay in HFF cells or neutral red reduction in Vero 76 cells

Specific Goal #2: To Assess the Oral Bioavailability, Pharmacokinetics and Toxicity of the Optimized Prodrugs of Cidofovir in Rodents, *in vivo.*

To measure the oral bioavailablilty, plasma and tissue pharmacokinetics of the alkyloxyalkanol derivatives of CDV, we contracted with Moravek Biochemicals of Brea, CA to synthesize the following compounds each having a stable radiolabel in the pyrimidine ring of cytosine:

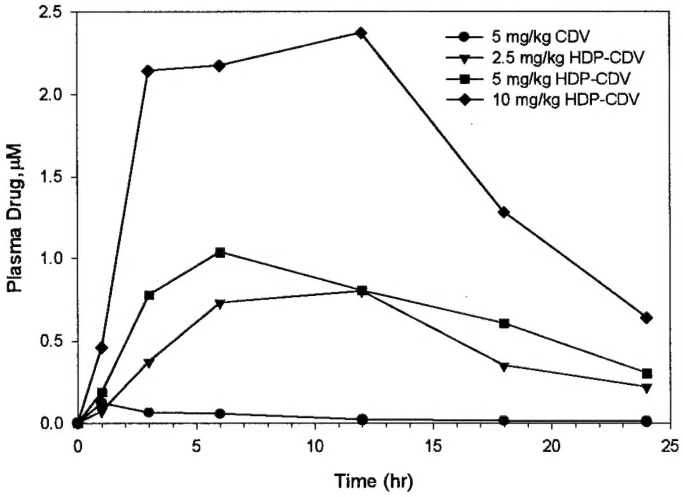
1. 1-O-Hexadecyloxypropyl-[2-^{14}C]cidofovir
2. 1-O-Octadecyloxyethyl-[2-^{14}C]cidofovir
3. 1-O-Oleyloxypropyl-[2-^{14}C]cidofovir

Our laboratory provided them with the nonradioactive alkyloxyalkyl bromides for the reactions and Dr. Moravek followed our method to couple the unlabeled ether lipid analogs to cyclic cidofovir to give the cyclic cidofovir equivalents. We then opened the ring with sodium hydroxide and carried out *in vivo* experiments as follows. All of the compounds have been received. We have done detailed studies to date only with #1.

2.1 Oral Bioavailability and Plasma and Tissue Pharmacokinetic Measurements:

We gave 2.5, 5, 10 mg/kg of HDP-[^{14}C]CDV or 5 mg/kg of [^{14}C]CDV to normal mice We obtained plasma at 1,3,6,12,18 and 24 hours and the data was plotted as □M drug versus time.

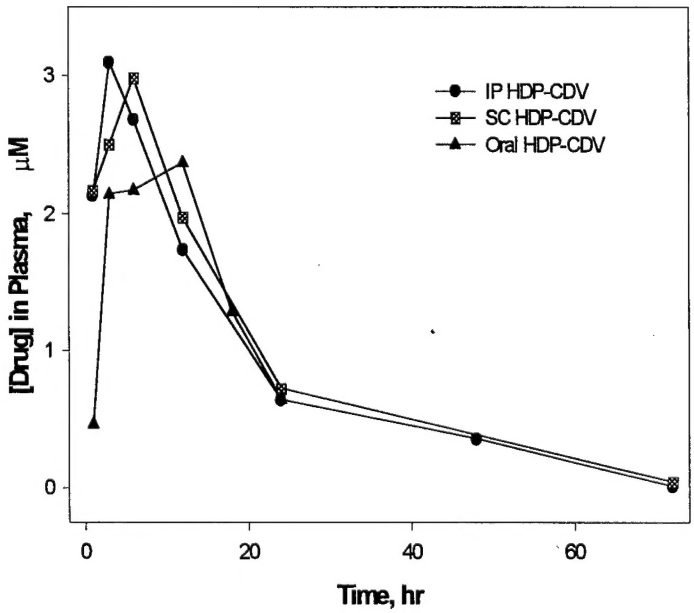
Figure 2.1.1. Oral Administration of HDP-CDV or CDV to Mice: Plasma Drug Levels



This experiment shows excellent plasma levels and roughly proportional area under curve values for HDP-CDV in plasma. Oral CDV (circles) is poorly absorbed as expected from the literature. Five and 10 mg/kg HDP-CDV gave peak plasma levels of 1 to 2 □M, repectively, well above the *in vitro* EC_{90} values for variola, vaccinia and cowpox (Section 1.1).

We then gave 10 mg/kg of HDP-[2-^{14}C]CDV orally, subcutaneously and intraperitoneally to mice and sampled plasma at 1,3,6,12,24,48 and 72 hours. The results are shown below:

Figure 2.1.1 Oral Administration of HDP-[2-^{14}C]CDV to Mice by Various Routes: Determination of Oral Bioavailability



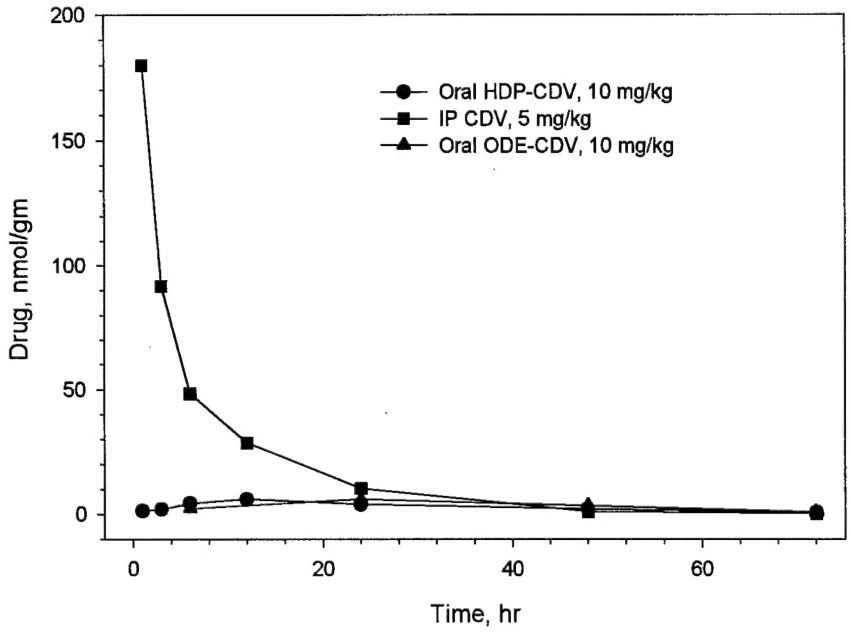
The parenterally administered HDP-[^{14}C]CDV showed higher peak values of 2.9 to 3.1 □M than seen with oral HDP-CDV. Using the formula for oral bioavailability, [AUC(parenteral)/AUC(oral)] x 100, and using the area under curve from zero to 72 hours, the average oral bioavailability is calculated to be 93% for HDP-[^{14}C]CDV. The oral bioavailability of unmodified cidofovir is <6% according to literature values reported previously by investigators from Gilead Sciences.

During the 02 year, we will do oral pharmacokinetic and bioavailability studies with 1-O-octadecyloxyethyl-[^{14}C]CDV (ODE-CDV), 1-O-oleyloxypropyl-[^{14}C]CDV and HDP-cyclic [^{14}C]CDV by the methods shown above.

2.2. Levels of Drug in Key Tissues Following Oral Administration of HDP-[^{14}C]CDV and ODE-[^{14}C]CDV.

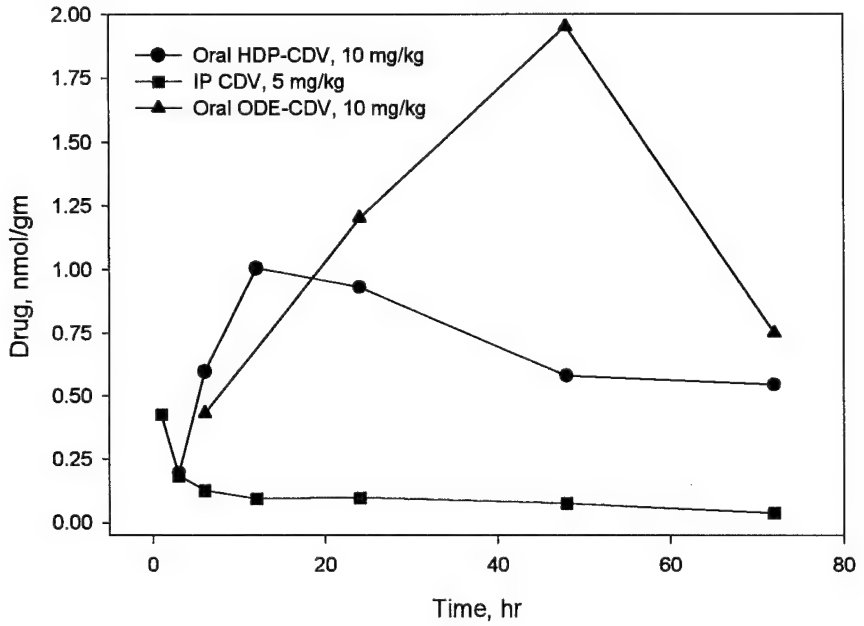
Since the kidney toxicity is the dose limiting toxicity for parenteral cidofovir (Vistide®), we treated mice with oral HDP-[^{14}C]CDV , ODE-[^{14}C]CDV or intraperitoneal [2-^{14}C]CDV at equimolar doses. We removed kidney at various times and determined the amount of radioactive drug present per gram of tissue.

Figure 2.2.1. Kidney Levels of HDP-CDV, ODE-CDV and CDV following oral or intraperitoneal administration to mice at equimolar doses (10 or 5 mg/kg)



The area under curve in kidney for for intraperitoneal CDV was 950 nmol.gm.hr versus 150 nmol.gm.hrs HDP-CDV or ODE-CDV given orally at a roughly equivalent molar dose. Thus, 6 times more i.p. CDV goes to kidney with than with oral HDP-CDV or ODE-CDV.

Figure 2.3.1. Lung Levels of HDP-CDV, ODE-CDV and CDV following oral or intraperitoneal administration to mice at equimolar doses (10 or 5 mg/kg)



We carried out similar measurements in lung because of the highly important role of poxviruses in producing pneumonitis. High levels of drug in lung would be beneficial in reducing viral titers in lung as much as possible. As shown above, HDP-CDV and ODE-CDV given orally lead to much higher levels of total drug in lung over the first 72 hours than intraperitoneal CDV at a roughly equimolar dose. Elimination from lung is much slower with the ether lipid analogs, possibly due to less rapid conversion to CDV and the mono- and diphosphate analogs or to a longer half life of the intracellular cidofovir mono- and diphosphates as we showed in MRC-5 human lung cells.

Preliminary reports from our collaborators at USAMRIID (not yet received in writing) and the data of Dr. Mark Buller in St. Louis indicate lower viral titers in lung, liver and spleen with oral drug treatment vs. parenteral cidofovir. Dr. Don Smee at Utah State University also reports significantly lower viral titers in lung with 100 mg/kg once or 3 mg/kg x 5 days.

Studies like those shown in Figure 2.3.1 will help with computer modeling of dosing regimens. The long intracellular half life of the lipid derivatized CDV will require carefully constructed dosing schedules to prevent rapid tissue build up of drug to levels which might be potentially toxic. Such studies are being done by Chimerix Inc., the licensee of the technology.

Specific Goal #3. To Evaluate Antiviral Activity of Selected Prodrugs of Cidofovir After Oral Administration to Animals Infected with Vaccinia Virus or Other Orthopox Viruses.

The raw data for all of the animal studies has been placed in the APPENDIX to this grant. In this section, I will present the main conclusions which can be reached from the respective studies of Vaccinia and Cowpox lethal challenge in Mice.

3.1 Studies with Cowpox at USAMRIID by Dr. John Huggins and coworkers

The raw data from a series of lethal cowpox challenge experiments in mice was provided by Dr. Robert O. Baker and Dr. John Huggins and is included in its entirety in the Appendix. For the purposes of the progress report I will present the most pertinent highlights from the results:

Table 3.1.1. Oral HDP-CDV in Lethal Aerosol Cowpox Infection:

Dose		Survivor/Tot	%Survival
HDP-CDV 20 mg/kg QD		9/10	90%
HDP-CDV 10 mg/kg QD		10/10	100%
HDP-CDV 5 mg/kg QD		10/10	100%
HDP-CDV 2.5 mg/kg QD	8/10		80%
HDP-CDV 1.25 mg/kg QD	7/10		70%
HDP-CDV 0.625 mg/kg QD	5/10		50%

In this study, full protection was obtained with 5 and 10 mg/kg as a single dose given daily for 5 days, starting 4 hours after infection. However, when the dose was divided into twice daily dosing, survival was slightly decreased suggesting that daily dosing is preferable. Drug toxicity was also assessed by treating animals which were uninfected with 5 daily doses of HDP-CDV: 20mg/kg (8/8); 10 mg/kg (18/18) and 5 mg/kg (18/18). All uninfected animals treated with drug survived.

Table 3.1.2. Oral HDP-CDV in Lethal Intranasal Cowpox Infection:

Dose	Survivor/Tot	%Survival
HDP-CDV 20 mg/kg QD	9/10	90%
HDP-CDV 10 mg/kg QD	10/10	100%
HDP-CDV 5 mg/kg QD	10/10	100%
HDP-CDV 2.5 mg/kg QD	8/10	80%
HDP-CDV 1.25 mg/kg QD	7/10	70%

In this study, intranasal infection followed 4 hours later by drug treatment showed full survival with 10 mg/kg HDP-CDV x5. 18 or 20 animals given 5 mg/kg/day for 5 days survived. When 10 or 5 mg/kg was given twice a day, 100% survival was noted.

Table 3.1.3. Single Oral Dose HDP-CDV in Lethal Intranasal Cowpox Infection

Dose	Day	Survivor/Tot	%Survival
HDP-CDV 140 mg/kg	-1	8/10	80%
HDP-CDV 140 mg/kg	0	1/10	10%
HDP-CDV 140 mg/kg	1	4/10	40%
HDP-CDV 140 mg/kg	2	8/10	80%
HDP-CDV 140 mg/kg	3	2/10	20%
HDP-CDV 70 mg/kg	0	7/10	70%
HDP-CDV 35 mg/kg	0	5/10	50%
HDP-CDV 18 mg/kg	0	2/10	20%
No Treatment	0	2/29	7%
HDP-CDV 140 mg/kg	Uninfected	10/10	100% [Tox control]

Single doses of oral HDP-CDV gave variable results. On day -1 (24 hrs before infection) 80% of animals survived with 140 mg/kg. However, if the same dose was given on day 0 (4 hours after infection) only 10% survived. Variable protection was noted with a single dose of 140 mg/kg on days 1, 2 and 3 after infection: 40,80,and 20%, respectively. A dose response was noted with single doses 4 hours after infection of 70, 35 and 18 mg/kg: 70, 50 and 20%. The single dose results are inferior to the 5 daily dose schedule shown above.

Dr. Baker and Dr. Huggins also did some comparisons of four of the most promising alkoxylalkyl analogs of CDV given orally in lethal intranasal cowpox infection.

Table 3.1.4. Effect of Treatment Delay with Oral HDP-CDV on Mortality from Lethal Intranasal Infection with Cowpox Virus.

Dose	Rx Days	Survivor/Tot	%Survival
HDP-CDV 10 mg/kg	0-4	10/10	100%
HDP-CDV 10 mg/kg	1-5	10/10	100%
HDP-CDV 10 mg/kg	2-6	9/10	90%
HDP-CDV 10 mg/kg	3-7	6/10	60%
HDP-CDV 10 mg/kg	4-8	5/10	50%

Delaying treatment with HDP-CDV, 10 mg/kg daily for 5 days, for 1 or 2 days following infection gave 90 to 100% survival. Waiting 3 or 4 days after infection to begin treatment gave lower survival, 50 to 60%.

Table 3.1.5. Comparison of 4 Different Analogs of CDV: HDP-; ODE-; OLP- and OLE-CDV in Lethal Intranasal Cowpox Infection.

		Survivors/Total			
Dose	Rx Days	HDP-CDV	ODE-CDV	OLP-CDV	OLE-CDV
10 mg/kg	0-4	20/20	10/10	10/10	10/10
5 mg/kg	0-4	18/20	10/10	10/10	10/10
2.5 mg/kg	0-4	8/10	10/10	7/10	9/10

These studies suggest that all 4 analogs are highly effective. ODE-CDV appears to be the most effective with 100% survival at 2.5 mg/kg for 5 days. At this dose, the other compounds gave 70 to 90% survival.

In summary, 5 daily doses of HDP-CDV provided full protection at 10 or 5 mg/kg when given 4 hours to 24 hours after infection. Protection diminished if treatment was delayed for 3 or 4 days but 50% or more survived even with long treatment delays. ODE-CDV appeared to be slightly more effective than HDP-CDV but the difference may not be statistically significant. Oleyloxypropyl or oleyloxyethyl analogs were also highly effective.

3.2 Studies with Cowpox and Vaccinia by Dr. Earl R. Kern and coworkers, University of Alabama, Birmingham.

The raw data from a series of lethal cowpox an vaccinia virus challenge experiments in mice was provided by Dr. Earl R. Kern of the University of Alabama, Birmingham, and is included in its entirety in the Appendix. For the purposes of the progress report I will present the most pertinent highlights from Dr. Kern's results:

In the first experiment, Dr. Kern evaluated oral HDP-CDV in 5 daily doses of 3, 1, 0.3 and 0.1 mg/kg versus intraperitoneal CDV at the same doses, starting 48 hours after infection with the viruses (intranasal infection).

Table 3.2.1. Effect of Oral HDP-CDV Given 48 hours Following Infection on Mortality from Cowpox or Vaccinia Virus

	Mortality/total number	
Drug/ dosage	Cowpox	Vaccinia
CDV 3 mg/kg ip	1/15***	12/14
CDV 1 mg/kg ip	1/15***	15/15
CDV 0.3 mg/kg ip	3/15**	15/15
CDV 0.1 mg/kg ip	8/15*	15/15
HDP-CDV 3 mg/kg oral	3/15**	15/15
HDP-CDV 1 mg/kg oral	9/15	15/15
HDP-CDV 0.3 mg/kg oral	15/15	15/15
HDP-CDV 0.1 mg/kg oral	14/15	15/15
Placebo	14/15	15/15

* $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs placebo

These experiments show that HDP-CDV given orally at 3 mg/kg for 5 days is effective against cowpox (20% mortality/80% survival). Intraperitoneal CDV is more active than the oral drug. Neither ip CDV nor oral HDP-CDV has any activity against vaccinia virus challenge.

The studies shown below in Table 3.2.2 demonstrate that all oral analogs have activity against lethal cowpox virus challenge, even 72 hours after infection. The most active oral compound is ODE-CDV which lowered mortality to 23% at doses of 6.7 mg/kg x5 (+24 hr) and 20% at 2 mg/kg x5 (+48hr). These differences were highly significant, $p<0.001$, versus placebo. Both HDP-CDV and ODE-CDV produced statistically significant lowering of mortality at 72 hr (47 & 54%, $p<0.01$ and $p<0.02$). At 20 mg/kg x5days, Dr. Kern found that all of the compounds were ineffective, due to apparent drug toxicity. CDV was highly effective by intraperitoneal administration at 6.7 mg/kg.

Table 3.2.2. Comparison of Oral HDP-CDV, ODE-CDV, OLE-CDV and OLP-CDV against Lethal Cowpox Virus Infection as a Function of Treatment Delay After Infection versus Intraperitoneal CDV

		% Mortality	
Drug	Rx Delay	2.0 mg/kg	6.7 mg/kg
HDP-CDV oral	+24	93	40***
	+48	100	86
	+72	100	47**
ODE-CDV oral	+24	36***	23***
	+48	20***	43**
	+72	86	54*
OLP-CDV oral	+24	93	86
	+48	100	29***
	+72	100	86
OLE-CDV oral	+24	53**	53**
	+48	80	33***
	+72	100	79
I.P. CDV	+24	53**	0***
	+48	73	0***
	+72	100	33***

* p 0.02; ** $p<0.01$; *** $p<0.001$;

Placebo 100% mortality

This table suggests that the most potent oral drugs are ODE-CDV > HDP-CDV. At 2 mg/kg for 5 days 24 hrs after infection ODE-CDV reduced mortality to 36% while 6.7 mg/kg reduced mortality to 23% (both $p<0.001$ versus placebo). IP Cidofovir at 2 mg/kg was less effective. With treatment delays of 48 to 72 hrs, the effect waned to 54% mortality with 6.7 mg/kg ($p<0.02$). HDP-CDV at 6.7 mg/kg reduced 24 hour treatment delay mortality to 40% ($p<0.001$) and 72 hr treatment delay mortality to 47% ($p<0.01$). Intraperitoneal CDV at 6.7 mg/kg was most effective. Doses of 20 mg/kg for 5 days was ineffective, presumably due to drug toxicity in Dr. Kern's laboratory.

Like the USAMRIID studies, the results from Dr. Kern suggest the ODE-CDV is more active than HDP-CDV.

3.3 Studies with Ectromelia virus (Mousepox) by Dr. R. Mark Buller, St. Louis University

Studies carried out in mousepox infection by Dr. R. Mark Buller also support the oral activity of HDP-, ODE-, OLP- and OLE-CDV.

Table 3.3.1 Ectromelia virus aerosol challenge of A/J mice treated with intraperitoneal CDV or 30 mg/kg of various alkoxyalkyl esters of CDV (Data courtesy of Dr. R. Mark Buller)

Drug	Drug ID	Morbidity on day 7 pi[2]	DOD	Mean DOD +/- STDEV	Mortality at day 21 pi
CDV	CDV	4-++	8, 10	9±1.41	2/4 (50%)
HDP-CDV	KA115-124A	4-+	21	21	1/4 (25%)
ODE-CDV	KA115-124B	4-+++	8, 9, 9, 12	9.5±1.73	4/4 (100%)
OLP-CDV	KA115-124C	4-0	NA	NA	0/4 (0%)
Control	(dH2O)	4-5+	7, 7, 7, 7	7±0	4/4 (100%)

[1]Mice were treated daily with 30mg/kg of drug by gavage on days -1 through 3. Infection by aerosol occurred on T=0. The presented aerosol dose was ~1.3 x 10^6 PFU.
[2]morbidity=0, healthy, no signs of sickness; +, face fur ruffled, no conjunctivitis; ++, face and body fur ruffled, hunched posture, eyes starting to look swollen; +++, face and body fur ruffled, hunched posture, both eyes have conjunctivitis; ++++, near death—same as above, plus eyes swollen shut, little or no movement, marked respiratory distress; +++++, dead.

Three alkoxyalkyl esters of cidofovir were also tested in an ECTV aerosol challenge model in A/J mice. In the pilot study groups of 4 A/J mice were treated once daily for 5 consecutive days with the indicated compounds at a dose of 30 mg/kg (0.54 mg/100µl per mouse per day by gavage). The mice were challenged on T=0 with a presented aerosol dose of 2 x 10^7 PFU of ECTV. The experiment was terminated at T=21 days post infection (pi). The mortality rate and Day of death are summarized. On sacrifice, the surviving mice treated with CDV looked sick. The surviving mice treated with HDP-CDV looked healthier that the CDV treated mice, but still not completely healthy. OLP-CDV treated mice looked completely normal at sacrifice.

Although these are small studies, the results are generally similar to previously reported data. Of note, the ODE-CDV compound had less activity than that of HDP-CDV or OLP-CDV. Mark recently reported that only 1 of 4 mice treated with HDP-CDV and 0 of 4 treated with OLE-CDV had residual virus in liver or spleen at 21 days versus 2 of 2 in the parenteral CDV-treated group. These pilot studies in Mark Buller's lab are highly promising and we will supply him with more compound for further animal trials in the near future.

Table 3.3.2 Ectromelia Virus Aerosol Challenge in A/J Mice Treated with 30 mg/kg Oral HDP-CDV (Data courtesy of Dr. R. Mark Buller)

Drug[1]	Drug ID#	Morbidity on day 7 pi[2]	DOD	Mean DOD ± STDEV	Mortality at day 16 pi
CDV	CDV	11-++	9,9,10	9.3±0.5	9/11[3] (82%)
HDP-CDV	KA116-46A	11-+	NA[4]	NA	0/11(0%)
ODE-CDV	KA116-46B	6-+++ 5-++++	9	NA	10/11[3] (91%)
OLP-CDV	KA116-46C	11-+	NA	NA	0/11 (0%)
OLE-CDV	KA116-46D	11-++	9	NA	1/11 (9%)
Control	(dH_2O)	1-++++ 3-++	7,7,7,7,7,7,7, 8,8,8,	7.3±0.5	11/11[3] (100%)

[1]Mice were treated daily with 30mg/kg drug by gavage on days 0 through 4. Infection by aerosol occurred ~ 4 hrs prior to the first dose of drug. The presented aerosol dose was 6.4×10^4 PFU.
[2]morbidity=0, healthy, no signs of sickness; +, face fur ruffled, no conjunctivitis; ++, face and body fur ruffled, hunched posture, eyes starting to look swollen; +++, face and body fur ruffled, hunched posture, both eyes have conjuctivitis; ++++, near death-same as above, plus eyes swollen shut, little or no movement, marked respiratory distress (mice with this level of sickness are euthanized).
[3]mice euthanized due to morbidity: CDV 6/11; ODE-CDV 9/11; and control 1/11.
[4]not applicable.

This experiment shows that full protection from lethal ectromelia challenge is provided by 30 mg/kg of HDP-, OLP- or OLE-CDV. 91% of animals treated with ODE-CDV died. We believe that this is probably due to drug toxicity, rather than lack of antiviral activity since a high percent of animals treated with 10 mg/kd x 5 days survived (see below).

Table 3.3.3. Tissue Viral Titers Following Ectromelia Virus Aerosol Challenge of A/J Mice treated with 30 mg/kg of Cidofovir and Alkoxyalkyl Esters of CDV for 5 Days (Data courtesy of Dr. R. Mark Buller)

		Virus infectivity PFU/ml[1]		
Drug	Drug ID#	Spleen	Liver	Lung
CDV	CDV	$1.7\text{x}10^7 \times\div .03^2$	$3.2\text{x}10^5 \times\div 0.07$	$1.3\text{x}10^4 \times\div 0.2$
HDP-CDV	KA116-46A	$<1\text{x}10^2$	$<1\text{x}10^2$	$4.3\text{x}10^3 \times\div 7.4$ (1)[3]
ODE-CDV	KA116-46B	$<1\text{x}10^2$	$<1\text{x}10^2$	$1.8\text{x}10^3 \times\div 1.2$ (2)
OLP-CDV	KA116-46C	$<1\text{x}10^2$	$<1\text{x}10^2$	$4.0\text{x}10^3 \times\div 16$ (1)
OLE-CDV	KA116-46D	$<1\text{x}10^2$	$<1\text{x}10^2$	$1.3\text{x}10^3 \times\div 8.1$ (1)
Control	dH_2O	$8.8\text{x}10^6 \times\div 13$	$1.7\text{x}10^7 \times\div 2.0$	$1.1\text{x}10^6 \times\div 0.3$

[1]Five mice from each treatment group were sacrificed at 7 days post aerosol challenge, lung, spleen and liver tissue was isolated. Each sample was ground in PBS (10%w/v), frozen and thawed three times, and sonicated for 20 seconds. The suspension was titrated on BS-C1 monolayers.
[2]Geometric means were calculated from sample PFU/ml values above the limit of detection.
[3]The value in the brackets indicates the number of samples in which infectivity was below the limit of detection ($<10^2$).

In Table 3.3.3. the viral infectivity titers in various tissues were determined in homogenates from liver, spleen and lung. All drugs except oral CDV reduced viral infectivity to undetectable levels in liver and spleen ($<1\text{x}10^2$ pfu/ml). Viral infectivity in lung was reduced by about 2.5 to 3 logs.

Table 3.3.4. Ectromelia Virus Aerosol Challenge of A/J Mice treated with 10 mg/kg of Cidofovir and Alkoxyalkyl Esters of CDV for 5 Days (Data courtesy of Dr. R. Mark Buller)

Drug[1]	Drug ID#	Morbidity on day 7 pi[2]	DOD	Mean DOD ± STDEV	Mortality at day 16 pi
CDV	CDV	8-+++ 3-++++	8,8,8,8,8,9,9	8.3±0.5	11/11[3] (100%)
HDP-CDV	KA116-46A	11-+	15,15	15±0	2/11(18%)
ODE-CDV	KA116-46B	11-+	15	NA[4]	1/11 (9%)
OLP-CDV	KA116-46C	11-++	16	NA	1/11 (9%)
OLE-CDV	KA116-46D	5-+ 6-++	15	NA	1/11 (9%)
Control	(dH_2O)	6-+++ 1-++++	7,7,7,7,8,8,8,8	7.5±0.5	11/11[3] (100%)

[1]Mice were treated daily with 10mg/kg of drug by gavage on days 0 through 4. Infection by aerosol occurred ~4 hrs prior to the first drug. The presented aerosol dose was 2.6×10^6 PFU.
[2]morbidity=0, healthy, no signs of sickness; +, face fur ruffled, no conjunctivitis; ++, face and body fur ruffled, hunched posture, eyes starting to look swollen; +++, face and body fur ruffled, hunched posture, both eyes have conjuctivitis; ++++, near death-same as above, plus eyes swollen shut, little or no movement, marked respiratory distress (mice with this level of sickness are euthanized).
[3]mortality includes euthanasia due to morbidity: CDV 4/11; control 3/11.
[4]not applicable.

Table 3.3.5. Virus infectivity levels in tissues of ectromelia virus aerosol challenged A/J mice treated with 10 mg/kg of cidofovir and alkoxyalkyl esters of cidofovir for five days

		Virus infectivity PFU/ml[1]		
Drug	Drug ID#	Spleen	Liver	Lung
CDV	CDV	$4.4x10^5 \times\div 20^2$	$3.6x10^6 \times\div 3.6$	$7.2x10^5 \times\div 7.6$
HDP-CDV	KA116-46A	$<1x10^2$	$<1x10^2$	$5.0x10^4 \times\div 23$ (3)[3]
ODE-CDV	KA116-46B	$<1x10^2$	$<1x10^2$	$1.1x10^6 \times\div 2.1$
OLP-CDV	KA116-46C	$<1x10^2$	$<1x10^2$	$1.6x10^4 \times\div 3.5$
OLE-CDV	KA116-46D	$<1x10^2$	$<1x10^2$	$9.6x10^4 \times\div 9.6$
Control	dH_2O	$2.0x10^6 \times\div 0.7$	$5.6x10^6 \times\div 0.008$	$8.5x10^4 \times\div 34$

[1]Five mice from each treatment group were sacrificed at 7 days post aerosol challenge, lung, spleen and liver tissue was isolated. Each sample was ground in PBS (10%w/v), frozen and thawed three times, and sonicated for 20 seconds. The suspension was titrated on BS-C1 monolayers.
[2]Geometric means were calculated from sample PFU/ml values above the limit of detection.
[3]The value in the brackets indicates the number of samples in which infectivity was below the limit of detection ($<10^2$).

These two tables (3.3.4 and 3.3.5) indicate that 10 mg/kg oral treatment with HDP-, ODE-, OLP- or OLE-CDV results in substantial survival, 82 to 91%, in a lethal ectromelia virus aerosol challenge. All drugs, with the exception of cidofovir, given orally at this dose, reduced spleed and liver virus infectivity titers to undetectable ($<1 \times 10^2$ pfu/ml). Viral titers in lung were variable versus control (which was lower than usual).

An experiment of the same design at doses of 3 mg/kg daily for 5 days is in progress in Dr. Buller's laboratory.

Ectromelia virus aerosol challenge of A/J mice treated with 3 mg/kg of cidofovir and alkoxyalkyl esters of cidofovir for five days

Drug[1]	Drug ID#	Morbidity[2] on day 7 pi[1]	DOD	Mean DOD ± STDEV	Mortality at day 21 pi
CDV	CDV	7-+++ 2-+	7,7,7,8,8,8,11,16,16	9.77±3.5	11/11[3] (100%)
HDP-CDV	KA116-46A	9-++ 2-+	11,12,14,14	12.75±1.30	8/11[3] (72%)
ODE-CDV	KA116-46B	11-+	12	NA[4]	1/11 (9%)
OLP-CDV	KA116-46C	11-++	9,11,13,19,20	14.4±4.36	9/11[3] (81%)
OLE-CDV	KA116-46D	11-+	9,11,14,14,19	13.4±3.38	6/11[3] (54%)
Control	(dH_2O)	7-+++	6,6,6,6,6,6,7,7	6.25±0.43	11/11[3] (100%)

[1]Mice were treated daily with 3mg/kg of drug by gavage on days 0 through 4. Infection by aerosol occurred ~4 hrs **prior** to the first drug treatment The presented aerosol dose was 2.0×10^6 PFU.
[2]morbidity=0, healthy, no signs of sickness; +, face fur ruffled, no conjunctivitis; ++, face and body fur ruffled, hunched posture, eyes starting to look swollen; +++, face and body fur ruffled, hunched posture, both eyes have conjuctivitis; ++++, near death-same as above, plus eyes swollen shut, little or no movement, marked respiratory distress (mice with this level of sickness are euthanized).
[3]mortality includes euthanasia due to morbidity: CDV 2/11; HDP-CDV 4/11; OLP-CDV 4/11; OLE-CDV 1/11, Control 3/11.
[4]not applicable.

At 3 mg/kg daily for 5 days, ODE-CDV is highly effective with mortality of 1/11. OLE-CDV is somewhat effective with 6/11 and HDP-CDV and OLP-CDV are ineffective with mortality of 8/11 and 9/11 animals, respectively.

Viral infectivity in spleen, lung and liver is pending.

In general Dr. Buller's data show that ODE-CDV is the most effective, providing >90% survival at 3 and 10 mg/kg daily for 5 days. The other compounds HDP-, OLP- and OLE-CDV are also highly effective at higher doses of 30 or 10 mg/kg for 5 days. ODE-CDV appears to be toxic at 30 mg/kg daily for 5 days. In light of what we now know about the prolonged plasma and tissue half life of these drugs, the doses we are giving can, in all likelihood, be reduced greatly while preserving good efficacy. This would be done by using a single loading dose followed by 3 or 4 daily maintanence doses 20 to 30% of the loading dose.

We believe that HDP-CDV and ODE-CDV should move into toxicokinetic studies in two species and that the pre-IND activities for these two compounds should be accelerated.

3.4 Studies with Cowpox and Vaccinia Viruses by Dr. Don Smee, State University of Utah

The complete data for these studies has been included in the Appendix materials courtesy of Dr. Don Smee.

3.4.1. Expt. NIA-282. Effects of once daily oral treatments with HDP-CDV compared to intraperitoneal or oral treatments with cidovoir on lethal cowpox virus respiratory infection in mice. Data of Dr. Don Smee.

The raw data is in the Appendix materials. This experiment found no increase in survival from oral treatment with 0.1, 0.3, 1 or 3 mg/kg of HDP-CDV or 30 mg/kg oral CDV. Doses of oral HDP-CDV of 1 or 3 mg/kg did increase the mean day of death from 8.6 to 10.1 and 11.4, respectively ($p<0.05$ or $p<0.01$)

3.4.2. Expt. NIA-284. Effects of a single oral dose of HDP-CDV compared to intraperitoneal treatment with CDV on lethal Cowpox infection in mice.

This experiment compared 100 mg/kg of oral HDP-CDV with intraperitoneal CDV at the same dose given once 24 hours after infection with cowpox. There were 7/10 survivors with oral HDP-CDV ($p<0.01$ vs placebo) versus 6/10 survivors with intraperitoneal CDV ($p<0.05$ vs placebo). Note that the molar dose of CDV is about twice that of HDP-CDV. Thus, this experiment suggests that a single oral dose of HDP-CDV is equivalent to a higher (molar) ip dose of CDV. Mean day of death increased with HDP-CDV from 8.6 to 11.3 ($p<0.01$) but did not change significantly with ip CDV. Lung viral titer was lowered by oral HDP-CDV by about 1.2 logs versus 0.6 logs with ip CDV.

As shown in the Appendix, Expt NIA-295, a similar experiment with the same design against lethal vaccinia challenge showed 8/10 survivors with 100 mg/kg ip CDV versus no survivors with oral HDP-CDV at the same dose.

KEY RESEARCH ACCOMPLISHMENTS: 01 Year

- Synthesis of >20 highly active esters of cyclic cidofovir and cidofovir which are more active than unmodified CDV against variola, monkeypox, cowpox, ectromelia (mousepox) and vaccinia viruses in vitro.

- Some of the most active and selective agents are HDP-CDV, ODE-CDV, OLP-CDV and OLE-CDV are >100 times more active against poxviruses.

- Demonstration of high oral bioavailability of HDP-CDV (93%) in mice using HDP-[2-^{14}C]CDV

- Demonstration that oral HDP-[2-^{14}C]CDV given orally does not concentrate in the mouse kidney. Kidney toxicity is dose-limiting for intravenous cidofovir.

- Demonstration that oral HDP-[2-^{14}C]CDV given orally produces higher drug levels in lung than produced by intraperitoneal [2-^{14}C]CDV in mice. The lung is a key therapeutic target tissue in poxvirus infections.

- Completed scale up synthesis of HDP-CDV (20 grams), ODE-CDV (10 gm), OLP-CDV (5 mg) and OLE-CDV (5 gm) and characterization of products by mass spectroscopy and NMR and HPLC. These compounds were used in animal efficacy studies in various model infections.

- Scale up synthesis of HDP-CDV and OLE-CDV to 50 to 100 gram levels is ongoing

- Oral HDP-CDV shown to provide complete protection from lethal cowpox challenge at 20, 10 and 5 mg/kg day x 5 days by USAMRIID investigators (Huggins)

- Oral HDP-CDV, ODE-CDV, OLP-CDV and OLE-CDV shown to have protective effects in lethal cowpox challenge even after treatment delays of up to 48 hours. 72 hour treatment delays provide less protection. (Huggins, Kern)

- Oral HDP-, ODE-, OLP- and OLE-CDV shown to provide 90+% protection at 10 mg/kg in lethal Ectromelia virus challenge in mice (mousepox). Viral titers in liver and spleen reduced to undetectable levels, lung levels generally decreased by this treatment (Buller).

- Discovered two new classes of analogs which are 6 to 80 times more active than HDP-CDV against poxviruses including cowpox, vaccinia, variola and monkeypox. These compounds will be further evaluated in the coming year.

REPORTABLE OUTCOMES: 01 Year

A. Abstracts presented at National or International Meetings:

1. Huggins, J.W., Baker, R.O., Beadle, J.R. and Hostetler, K.Y., Orally active ether lipid prodrugs of cidofovir for the treatment of smallpox, Antiviral Research, 53:A66(104), 2002; Oral presentation at ICAR, Prague, March, 2002

2. Winegarden, K.L., Ciesla, S.L., Aldern, K.A., Beadle, J.R. and Hostetler, K.Y., Oral pharmacokinetics and preliminary toxicology of 1-O-hexadecyloxypropyl-cidofovir in mice, March, 2002, Antiviral Research, 53:A67(105), 2002; Oral presentation at ICAR, Prague, March, 2002

3. Aldern, K.A., Ciesla, S.L., Winegarden, K.L. and Hostetler, K.Y., 1-O-Hexadecyloxypropyl-[^{14}C]cidofovir: cellular uptake and metabolism in MRC-5 human lung fibroblasts, *in vitro,* Antiviral Research, 53:A62 (92), 2002; Poster presentation at ICAR, Prague, March, 2002,

4. Wan, W.B., Beadle, J.R., Aldern, K.A. and Hostetler, K.Y., Alkoxyalkyl Esters of Cidofovir and cyclic cidofovir: effects of alkyl chain length, unsaturation and substitution on the in vitro antiviral activity in cells infected with HSV-1 and CMV, Presentation at the American Chemical Society, Medicinal Chemistry Meeting, Boston, MA, August 2002.

5. Wan, W.B., Beadle, J.R., Aldern, K.A., Keith, K., Kern, E.R. and Hostetler, K.Y., Alkoxyalkylesters of cidofovir with improved oral bioavailability and antiviral potency against cowpox and vaccinia viruses: In search of the optimal ester. Presentation at Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, September, 2002.

B. Manuscripts published or submitted

1. Kern, E.R., C. Hartline, C., Harden, E., Keith, K., Rodriguez, N, Beadle, J.R. and Hostetler, K.Y., Enhanced inhibition of orthopoxvirus replication in vitro by alkoxyalkyl esters of cidofovir and cyclic cidofovir, Antimicrobial Agents Chemotherapy, 46:991-995, 2002.

2. Aldern, K.A., Ciesla, S., Winegarden, K. and Hostetler, K.Y., The increased antiviral activity of 1-O-hexadecyloxypropyl-cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism, submitted for publication, Molecular Pharmacology, October, 2002.

C. Presentations Pending

1. Karl Y. Hostetler, "Design and Development of Drugs for Smallpox", Biodefense Research, Technologies and Applications: November 5, 2002, Hilton McLean Tysons Corner, McLean, VA.

D. Awards

1. "Oral Smallpox Drug", Winner, Popular Science Magazine's 2002 Best of Whats New Award. (Embargoed until November 8, 2002).

CONCLUSIONS:

In our first year of work under this Army grant, we synthesized over 20 active antiviral compound active against variola, vaccinia, monkeypox, cowpox and mousepox. Most of these compounds are substantially more active and selective than cidofovir. Our lead compounds, 1-O-hexadecyloxypropyl-cidofovir (HDP-CDV) and 1-O-octadecyloxypropyl-cidofovir (ODE-CDV), have excellent orally bioavailability. Furthermore, they have been shown to prevent death from lethal cowpox and mousepox viral challenge. In addition, some beneficial effects have been seen in lethal vaccinia challenge in mice, if treatment is given 4 to 24 hours after infection. Oral HDP-CDV and ODE-CDV given at 10mg/kg day for 5 days reduce mousepox virus levels to undetectable in liver and spleen, while providing 1 to 3 log reductions in lung. We have also identified two new classes of highly active anti-poxvirus drugs NOT based on cidofovir.

In the coming year of the project we will continue to support our collaborators at USAMRIID by providing larger quantities of the two most active lead compounds: HDP-CDV and ODE-CDV for advanced animal studies. We will also intensify our research on the two new compounds to determine oral bioavailability and activity in various animal models of poxvirus disease. We do not believe that any changes in general direction or activities is necessary beyond those described in the original proposal.

With regard to possible medical products, we believe that HDP-CDV or ODE-CDV represent excellent candidates drugs for oral prevention or treatment of smallpox. The best of these two drugs could be developed to IND filing and human safety testing in order to provide a therapy/preventative for immunosuppressed Americans or Americans with eczema or atopic dermatitis who cannot be safely vaccinated. Final proof of concept would require successful testing in an FDA acceptable primate model of smallpox like that currently being developed by Dr. Peter Jahrling and Dr. John Huggins of USAMRIID.

APPENDIX TO

"Development of Potent Orally Active Agents for Prevention and Treatment of Smallpox"
DAMD 17-01-2-0071

Karl Y. Hostetler, M.D.
Principal Investigator

October 27, 2002

Items in the Appendix:

A1. Submitted Manuscript: Aldern, K.A., Ciesla, S., Winegarden, K. and Hostetler, K.Y., The increased antiviral activity of 1-O-hexadecyloxypropyl-cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism, Submitted for publication, Molecular Pharmacology, 2002.

A2. Animal Results: Robert O. Baker and John R. Huggins, USAMRIID/CDC. Lethal Cowpox Virus challenge studies with Oral CDV Analogs

A3. Animal Results: Earl R. Kern, University of Alabama, Birmingham; Lethal Cowpox and Vaccinia Virus challenge data with Oral CDV analogs

A4. Animal Results: R. Mark Buller, St. Louis University; Lethal Ectromelia Virus Challenge Results with Oral CDV analogs

A5. Animal Results: Don Smee, Utah State University; Lethal Cowpox and Vaccinia Virus data with Oral CDV analogs

A6. Published Papers: Kern, E.R., C. Hartline, C., Harden, E., Keith, K., Rodriguez, N, Beadle, J.R. and Hostetler, K.Y., Enhanced inhibition of orthopoxvirus replication in vitro by alkoxyalkyl esters of cidofovir and cyclic cidofovir, Antimicrobial Agents Chemotherapy, 46:991-995, 2002.

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THE INCREASED ANTIVIRAL ACTIVITY OF 1-O-HEXADECYLOXYPROPYL-[^{14}C]CIDOFOVIR IN MRC-5 HUMAN LUNG FIBROBLASTS IS EXPLAINED BY UNIQUE CELLULAR UPTAKE AND METABOLISM

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ABSTRACT

Recently there has been renewed interest in finding orally active drugs against smallpox. Cidofovir (CDV) given by parenteral injection has been shown to protect against lethal poxvirus infection. We have been interested in the synthesis and evaluation of orally active derivatives of CDV. Previous studies showed that the CDV and cyclic cidofovir (cCDV) analogs, 1-O-hexadecyloxypropyl-CDV (HDP-CDV) and 1-O-hexadecyloxypropyl-cCDV (HDP-cCDV), show >100-fold increases in antiviral activity versus the unmodified nucleosides against cells infected with orthopoxviruses, cowpox and vaccinia virus. In contrast to CDV, HDP-CDV is orally bioavailable and has been reported to be orally active in lethal cowpox virus infection in mice. To assess the metabolic basis for the increased antiviral activity of HDP-CDV *in vitro*, we studied the cellular uptake and anabolic metabolism of ^{14}C-labeled CDV, cCDV and their alkoxyalkanol esters, HDP-CDV and HDP-cCDV. HDP-CDV and HDP-cCDV were taken up rapidly by MRC-5 human lung fibroblasts *in vitro* but uptake of CDV and cCDV was much slower. Analysis of cellular metabolites showed that levels of cidofovir diphosphate (CDV-DP), the active antiviral compound, were >100 times greater with HDP-CDV than levels observed with CDV. We found that the intracellular half life of CDV-DP was 10 days versus 2.7 days reported for CDV. HDP-CDV appears to circumvent poor cellular uptake by rapid association with cellular membrane phospholipids while CDV uptake proceeds via the slow process of fluid endocytosis.

INTRODUCTION

Cidofovir (1-[(S)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine; CDV) is an acyclic phosphonate analog of cytosine which has been shown to have activity against many DNA viruses including herpes group viruses, orthopoxviruses, adenovirus and varicella zoster virus (4, 5, 13). CDV (Vistide®; Gilead Sciences) is approved as an intravenous treatment for cytomegalovirus retinitis in AIDS patients but has dose-limiting renal side effects (11,12). CDV given intravenously protects mice against lethal cowpox virus infection (Bray et al, 2000).

It would be useful to have highly active antiviral analogs of CDV which are less toxic and orally bioavailable. Our laboratory has developed a strategy to enhance absorption of poorly absorbed nucleotides such as acyclovir monophosphate and ganciclovir monophosphate by attaching certain ether lipid residues such as 1-O-hexadecylpropanediol (3,7,8,9). As part of this program, we synthesized 1-O-hexadecyloxypropyl-CDV (HDP-CDV) and tested it against MRC-5 human lung fibroblasts infected with cytomegaloviruses, herpes simplex viruses, type-1 and type-2. HDP-CDV exhibited multiple log increases in antiviral activity *in vitro* against CMV and HSV-1 compared with CDV (2). HDP-CDV was also active against various ganciclovir-resistant CMV isolates (2). Multiple log enhancement of antviral activity was also noted against various strains of cowpox and vaccinia virus infected cells *in vitro* (10) and against variola virus- infected cells *in vitro* (J.W. Huggins, personal communication, 2000). In this paper we have compared the cellular uptake and intracellular metabolism of HDP-[2-^{14}C]CDV and [2-^{14}C]CDV to assess the mechanisms leading to the remarkable increase in antiviral activity observed in our prior studies.

MATERIALS AND METHODS

CELL UPTAKE STUDIES:

Radiolabeled CDV, cCDV, HDP-CDV or HDP-cCDV at concentrations of 1, 3 or 10 μM (specific activity, 50 to 56 μCi/μmol) were added to 24 well plates containing subconfluent monolayers of MRC-5 human lung fibroblast cells and incubated at 37°C for the times indicated. The medium was then removed and the cell monolayers were washed with cold phosphate buffered saline (PBS), lysed with (O.5 N) sodium hydroxide and transferred to scintillation vials for counting.

METABOLISM EXPERIMENTS:

[2-^{14}C]CDV or HDP-[2-^{14}C]CDV, 10 μM (specific activity 50 and 56 mCi/mmol, respectively) was added to 25 cm^2 flasks of near confluent MRC-5 cells and incubated for 6, 24 or 48 hr. The cell monolayers were treated as follows: the media was removed and the cell monlayer was washed twice with cold PBS. Then 0.6 ml of distilled water was added and the flasks were frozen and thawed twice followed by sonication in a cold bath sonicator for five minutes. The cells were removed by scraping and transferred to a glass tube. Cold trichloroactic acid was added to a final concentration of 8% and the contents vortexed and centrifuged for 10 minutes at 4°C. The supernatant was removed, counted and immediately analyzed by HPLC. HPLC was done by applying the sample to a 4.6 x 15 cm Partisil 10 SAX column with a SAX guard column. The column was eluted at a flow rate of 1 ml/min using a potassium phosphate buffer gradient of 20 mM to 700 mM, pH 5.8 beginning at 9 minutes for a duration of 20 minutes and a terminal hold of 5 minutes. One minute/ml fractions were collected and FloScint IV scintillation fluid added and the samples were analyzed by liquid scintillation counting.

For the drug wash-out experiments, cells were incubated for 24 hrs with HDP-[^{14}C]CDV. The media was removed and the cell monolayer washed two times with cold PBS. Drug-free complete media was added and the cells were incubated and harvested at 0, 2, 4, 6, 8 and 10 days and analyzed by Partisil SAX HPLC as noted above. **CELLS AND MEDIA :**

MRC-5 human lung fibroblasts were obtained from American Type Culture collection at an early pass number. Cells were grown in minimal essential medium with Earle's salts (MEM) containing 2% fetal bovine serum. Fetal bovine serum was obtained from Gibco BRL, Carlsbad, CA.

CHEMICALS AND RADIOCHEMICALS:

Cidofovir and cyclic cidofovir were provided by Gilead Sciences Inc., Foster City CA . [2-^{14}C]CDV (specific activity 53 mCi/mmol), cyclic[2-^{14}C]CDV (specific activity 56 mCi/mmol), and HDP-cyclic[2-^{14}C]CDV (specific activity 50 mCi/mmol) were prepared by custom synthesis by Moravek Biochemicals, Brea, CA. HDP-cyclic[2-^{14}C]CDV was treated with with dilute NaOH to open the ring and HDP-[2-^{14}C]CDV was isolated as the monosodium salt. Cidofovir monophosphate and cidofovir diphosphate were prepared by custom synthesis by TriLink BioTechnologies, San Diego, CA.

RESULTS

To assess drug uptake, MRC-5 cells were exposed to 10 μM CDV or HDP-CDV for times ranging from 1 to 24 hours and drug uptake was assessed. Cellular uptake of CDV was maximal at 1 to 4 hours but remained stable or declined slightly by 24 hrs. In contrast, the cellular drug content of HDP-CDV increased nearly linearly for 6 hr and progressively to 24 hrs (Figure 1). Similar results were observed with HDP-cCDV except that cellular drug levels stopped rising after 4 hours and declined slowly thereafter. The uptake of HDP-cCDV was about twice that observed with HDP-CDV. Cyclic CDV uptake was generally similar to that of CDV (Figure 1).

To assess the effect of concentration on drug uptake, we evaluated the cellular uptake of 1, 3 and 10 μM CDV, cCDV, HDP-cCDV and HDP-CDV at four hours during the linear phase of cellular drug uptake. At 4 hours, the cellular drug content observed with 1 μM CDV was 1.2 picomoles/well versus 28 picomoles/well with 1.0 μM HDP-CDV. At concentrations of 3 and 10 μM, drug uptake of HDP-CDV was 77 and 245 picomoles/well, an increase of 11 to 23-fold versus CDV (Figure 2). At 3 and 10 μM the uptake of HDP-cCDV was approximately twice that of HDP-CDV and 32-fold greater that observed with cCDV (Figure 2).

We next exposed cells to 10 μM drug for various times and evaluated the intracellular levels of CDV, CDV monophosphate (CDV-MP) and CDV diphosphate (CDV-DP). HPLC analysis of extracts of cells exposed to 10 μM HDP-CDV revealed readily detectable peaks at the same retention times as authentic standards of CDV-MP and CDV-DP. However, cellular levels of CDV-MP and CDV-DP were much lower in cells exposed to 10 μM CDV. After 24 hours, CDV-MP level was 1.0 picomole/flask with CDV versus 63 picomoles/flask with HDP-CDV. CDV-DP, the active antiviral, was 1.3 with CDV versus 133 with HDP-CDV,

an increase of 102-fold. After 48 hours, cellular CDV-DP was 1.8 picomoles/flask with CDV versus 184 with HDP-CDV, and increase of 102-fold (Table 2). Interestingly, we did not observe a radioactive peak eluting between CDV and CDV-MP, reported previously as CDV diphosphocholine by others (1,6). However, small amounts of a radioactive compound eluting before CDV were noted and seemed to correspond to (S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]uridine (HPMPU), the deamination product of CDV (HPMPC).

To assess the intracellular levels of the metabolites of HDP-[2-^{14}C]CDV, we exposed cells to 7.5 μM HDP-[2-^{14}C]CDV for 24 hours. Then the radioactive drug was washed away with PBS and the medium was replaced with drug free growth medium and incubation continued for 2 to 10 days. Cell metabolites were analyzed by HPLC at 0, 2, 4, 6, 8 and 10 days following removal of the drug. Cell extracts were prepared by freezing and thawing in water and the membrane fraction was isolated by centrifugation. The membrane fraction contained unmetabolized HDP-[2-^{14}C]CDV which represented the 2084 picomoles/flask at time zero. The water soluble metabolites consisted of CDV, CDV-MP and CDV-DP, 460, 45 and 83 picomoles/flask respectively at zero time (Figure 3). Two days after the washout of HDP-[2-^{14}C]CDV from the flask, membrane levels of HDP-[2-^{14}C]CDV declined by 52% while the water soluble metabolites, [2-^{14}C]CDV, [2-^{14}C]CDV-MP and [2-^{14}C]CDV-DP increased by 58%, 102% and 64%, reaching peak levels of 722, 74 and 166 picomoles/flask, respectively. Thereafter, CDV, CDV-MP and CDV-DP declined gradually to 267, 24 and 83 picomoles/flask at 10 days. The $T_{½}$ values for HDP-CDV, CDV, CDV-MP and CDV-DP were estimated to be 2, 8, 7 and 10 days, respectively (Figure 3).

DISCUSSION

Covalent addition of the 1-O-hexadecyloxypropyl ester to the phosphonate of CDV results in remarkable increases in the antiviral activity of HDP-CDV against HCMV and HSV (2) and against vaccinia virus and cowpox viruses (10). The present study indicates that this is due at least in part to increased cell penetration of HDP-CDV relative to CDV. Furthermore, the intracellular levels of the active antiviral metabolite, CDV-DP, formed after intracellular cleavage of HDP-CDV by phospholipase C-like enzymes and phosphorylation by cellular kinases is more than two logs greater that the levels observed with equimolar concentrations of CDV. The intracellular half life of CDV-DP is approximately 10 days with HDP-CDV versus a reported half life in cells exposed to CDV of 17 hours (6) or in Vero cells, where a biphasic decay of CDV-DP was observed with $T_{½}$ values of 1 and 2.7 days (1) . The ratios of CDV, to CDV-MP and CDV-DP observed when cells were exposed to HDP-CDV were generally similar to that seen with CDV alone as reported by Ho and coworkers (6) and Aduna et al (1), i.e. CDV>>CDV-DP>CDV-MP. Surprisingly, we did not observe conversion of CDV to CDV diphosphate choline in these experiments, in contrast to prior reports (1,6). An important cause of the 10 day $T_{½}$ value observed for CDV-DP following exposure of cells to HDP-CDV in this study, is the presence in MRC-5 cellular membranes of a large pool of HDP-CDV which is metabolized by cellular phospholipase C and phosphomonoesterases to release intracellular CDV which may be anabolized in turn to CDV-DP by cellular kinases.

In conclusion, the present study shows that the cellular uptake of HDP-CDV is 11 to 23-fold greater than that of CDV in MRC-5 cells in vitro. With 10 μM HDP-CDV, the intracellular level of the active antiviral, CDV-DP, was 102 times greater than that observed with CDV at both 24 hr and 48 hr. This would appear to

explain, at least in part, the multiple log increases in antiviral activity observed with HDP-CDV in CMV, HSV-1, cowpox and vaccinia virus infected cells in vitro. Finally, the intracellular half-life of CDV-diphosphate was 10 days in MRC-5 human lung fibroblasts exposed to HDP-CDV, suggesting that long lasting antiviral activity may be provided by relatively infrequent exposures of cells to drug. Because HDP-CDV and other compounds of this class are also orally bioavailable, at least in rodents, the compounds are worthy of further investigation as possible oral therapies for viral disease caused by susceptible viruses including HCMV, HSV and orthopoxviruses including smallpox (variola major) and vaccinia.

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TABLE 1. ACTIVITY OF CIDOFOVIR AND 1-O-HEXADECYLOXYPROPYL- CIDOFOVIR AGAINST HERPES GROUP VIRUSES AND POXVIRUSES, *IN VITRO*

	EC_{50}, µM, by Plaque Reduction			
Virus	**CDV**	**HDP-CDV**	**HDP-cCDV**	**Reference**
HCMV-AD169	0.38	0.0009 (422)	0.001 (380)	2
HCMV-Towne	0.40	0.0009 (444)	0.001 (400)	2
HSV-1	15.2	0.06 (253)	0.06 (253)	2
HSV-2	10.5	0.08 (131)	0.23 (46)	2
Cowpox-Brighton	44.7	0.60 (74)	2.10 (21)	10
Vaccinia-Copenhagen	46.2	0.80 (58)	3.80 (12)	10
Vaccinia-WR	45.8	1.10 (42)	5.60 (8)	10
Vaccinia-Elstree	41.6	1.20 (35)	3.80 (11)	10

Number in parentheses are the -fold increases in antiviral activity versus CDV. Data abstracted from References 2 & 10 and used with permission.

Abbreviations: CDV, cidofovir; HDP-CDV, 1-O-hexadecyloxypropyl-cidofovir; HDP-cCDV, 1-O-hexadecyloxypropyl-cyclic cidofovir; EC_{50}, 50% effective concentration.

TABLE 2. COMPARISON OF METABOLITE LEVELS FOUND IN MRC-5 CELLS FOLLOWING EXPOSURE TO [2-^{14}C]CDV (10 μM) OR HDP-[2-^{14}C]CDV (10 μM).

	CDV*			HDP-CDV*		
Metabolite	**6 hr**	**24 hr**	**48 hr**	**6 hr**	**24 hr**	**48 hr**
HPMPU	4.8	6.9	4.2	8.8	27.3	36.0
CDV	146.4	273.8	129.3	166.7	697.4	702.0
CDV-MP	3.9	1.0	1.2	11.8	63.2	71.4
CDV-DP	6.3	1.3	1.8	11.2	132.6	184.4

* - picomoles/flask

Abbreviations: HPMPU, (S)–1-[3-Hydroxy-2-(phosphonoylmethoxy)propyl]uridine; CDV, cidofivir; CDV-MP, cidofovir monophosphate; CDV-DP, cidofovir diphosphate.

A2. Animal Results: Robert O. Baker and John R. Huggins, USAMRIID/CDC; Lethal Cowpox Virus Challenge Studies with Oral CDV Analogs

Data Provided by Dr.Robert O. Baker , USAMRIID

Drug	Dose	Schedule	Viral route	Surv./Tot.	% survival	MTD
CDV oral	20 mg/kg QD	D 0-4	Aero	1/10	10%	11.1
	10 mg/kg BID	D 0-4	Aero	1/10	10%	11.1
	20 mg/kg QD	D 0-4	---	5/5	100%	---
	10 mg/kg BID	D 0-4	---	5/5	100%	---
HDP-CDV	20 mg/kg QD	D 0-4	Aero	9/10	90%	7.0
	10 mg/kg QD	D 0-4	Aero	20/20	100%	---
	5 mg/kg QD	D 0-4	Aero	20/20	100%	---
	2.5 mg/kg QD	D 0-4	Aero	8/10	80%	11.5
	1.25 mg/kg QD	D 0-4	Aero	7/10	70%	11.7
	0.63 mg/kg QD	D 0-4	Aero	5/10	50%	11.4
	10 mg/kg BID	D 0-4	Aero	19/20	95%	11.0
	5 mg/kg BID	D 0-4	Aero	20/20	100%	---
	2.5 mg/kg BID	D 0-4	Aero	5/10	50%	11.8
	1.25 mg/kg BID	D 0-4	Aero	3/10	30%	10.6
	0.63 mg/kg BID	D 0-4	Aero	0/10	0%	9.7
	20 mg/kg QD	D 0-4	---	8/8	100%	---
	10 mg/kg QD	D 0-4	---	18/18	100%	---
	5 mg/kg QD	D 0-4	---	18/18	100%	---
	10 mg/kg BID	D 0-4	---	18/18	100%	---
	5 mg/kg BID	D 0-4	---	18/18	100%	---
	2.5 mg/kg BID	D 0-4	---	8/8	100%	---
	10 mg/kg QD	D 0-4	i.n.	20/20	100%	---
	5 mg/kg QD	D 0-4	i.n.	18/20	90%	11.0
	2.5 mg/kg QD	D 0-4	i.n.	8/10	80%	8.0
	10 mg/kg BID	D 0-4	i.n.	10/10	100%	---
	5 mg/kg BID	D 0-4	i.n.	10/10	100%	---
	140 mg/kg	D -1	i.n.	8/10	80%	9.5
	140 mg/kg	D 0	i.n.	1/10	10%	7.3
	140 mg/kg	D 1	i.n.	4/10	40%	8.0
	140 mg/kg	D 2	i.n.	8/10	80%	8.0
	140 mg/kg	D 3	i.n.	2/10	20%	7.0
	70 mg/kg	D 0	i.n.	7/10	70%	10.0
	35 mg/kg	D 0	i.n.	5/10	50%	8.8
	18 mg/kg	D 0	i.n.	2/10	20%	9.1
	140 mg/kg	D 0	---	10/10	100%	---
	10 mg/kg QD	D 1-5	i.n.	10/10	100%	---
	10 mg/kg QD	D 2-6	i.n.	9/10	90%	10
	10 mg/kg QD	D 3-7	i.n.	6/10	60%	10.2
	10 mg/kg QD	D 4-8	i.n.	5/10	50%	9.5
	20/20/2.5 mg/kg	D –1/0/1-8	Aero	9/10	90%	8.0
	20/20/2.5 mg/kg	D –1/0/1-8	i.n.	8/10	80%	10.5
OLE-CDV	10 mg/kg QD	D 0-4	i.n.	10/10	100%	---
	5 mg/kg QD	D 0-4	i.n.	10/10	100%	---
	2.5 mg/kg	D 0-4	i.n.	9/10	90%	12.0

OLP-CDV	10 mg/kg QD	D 0-4	i.n.	10/10	100%	---
	5 mg/kg QD	D 0-4	i.n.	10/10	100%	---
	2.5 mg/kg	D 0-4	i.n.	7/10	70%	8.7
	20/20/2.5 mg/kg	D –1/0/1-8	Aero	10/10	100%	---
	20/20/2.5 mg/kg	D –1/0/1-8	i.n.	9/10	90%	10
ODE-CDV	10 mg/kg QD	D 0-4	i.n.	10/10	100%	---
	5 mg/kg QD	D 0-4	i.n.	10/10	100%	---
	2.5 mg/kg	D 0-4	i.n.	10/10	100%	---
	20/20/2.5 mg/kg	D –1/0/1-8	Aero	10/10	100%	---
	20/20/2.5 mg/kg	D –1/0/1-8	i.n.	9/10	90%	10
Placebo	---	D 0-4	Aero	1/17	5.9%	9.0
Placebo	---	D 0-4	i.n.	2/29	6.9%	9.1
Placebo	---	D 0-4	---	20/20	100%	---
---	---	---	Aero	0/15	0%	9.1

A3. Animal Results: Earl R. Kern, University of Alabama, Birmingham; Lethal Cowpox and Vaccinia Virus challenge data with Oral CDV analogs

Effect of Oral Treatment with ARB-00-394 (HDP-CDV) on the Mortality of BALB/c Mice Inoculated Intranasally with Cowpox Virus

	Mortality				
Treatment[a]	**Number**	**Percent**	**P-Value**	**MDD**	**P-Value**
Placebo Saline i.p. + 48 hr	14/15	93	- - -	12.1	- - -
CDV i.p. 3 mg/kg + 48 hr	1/15	7	<0.001	13.0	NS
1 mg/kg + 48 hr	1/15	7	<0.001	10.0	NS
0.3 mg/kg + 48 hr	3/15	20	<0.01	19.3	0.01
0.1 mg/kg + 48 hr	8/15	53	<0.05	10.4	<0.01
Placebo Saline p.o. + 48 hr	12/15	80	- - -	10.8	- - -
HDP-CDV p.o.					
3 mg/kg + 48 hr	3/15	20	<0.01	14.0	<0.01
1 mg/kg + 48 hr	9/15	60	NS	12.9	NS
0.3 mg/kg + 48 hr	15/15	100	NS	11.2	NS
0.1 mg/kg + 48 hr	14.15	93	NS	11.2	NS

[a] Treatment given once daily for 5 days.

Effect of Oral Treatment with ARB-00-394 (HDP-CDV) on the Mortality of BALB/c Mice Inoculated Intranasally with Vaccinia Virus

	Mortality				
Treatment[a]	**Number**	**Percent**	**P-Value**	**MDD**	**P-Value**
Placebo Saline i.p. + 48 hr	15/15	100	- - -	6.1	- - -
CDV i.p. 3 mg/kg + 48 hr	12/15	80	NS	8.8	<0.001
CDV i.p. 1 mg/kg + 48 hr	15/15	100	NS	6.7	<0.01
CDV i.p. 0.3 mg/kg + 48 hr	15/15	100	NS	6.3	NS
CDV i.p. 0.1 mg/kg + 48 hr	15/15	100	NS	6.5	<0.05
Placebo Saline p.o. + 48 hr	15/15	100	- - -	6.1	- - -
HDP-CDV Oral					
HDP-CDV 3 mg/kg + 48 hr	15/15	100	NS	6.1	NS
HDP-CDV 1 mg/kg + 48 hr	15/15	100	NS	6.1	NS
HDP-CDV 0.3 mg/kg + 48 hr	15/15	100	NS	6.4	NS
HDP-CDV 0.1 mg/kg + 48 hr	15.15	100	NS	6.4	NS

[a] Treatment given once daily for 5 days.

Effect of Oral Treatment with HDP-CDV, ODE-CDV, OLE-CDV, or OLP-CDV on the Mortality of BALB/c Mice Inoculated Intranasally with Cowpox, Brighton

	Mortality				
Treatment[a]	Number	Percent	P-value	MDD[b]	P-value
Placebo					
Saline +24h	15/15	100	---	9.7	---
CDV					
20 mg/kg +24h	2/15	13	<0.001	14.5	0.01
6.7 mg/kg +24h	0/15	0	<0.001	---	<0.001
2.0 mg/kg +24h	8/15	53	<0.01	13.4	0.001
Placebo					
Water +24h	15/15	100	---	9.3	---
HDP-CDV					
20 mg/kg +24h	15/15	100	NS	9.2	NS
6.7 mg/kg +24h	6/15	40	0.001	9.5	NS
2.0 mg/kg +24h	13/14	93	NS	12.5	<0.001
20 mg/kg toxicity	4/10	40	--	9.5	--
ODE-CDV					
20 mg/kg +24h	14/14	100	NS	7.4	<0.001
6.7 mg/kg +24h	3/13	23	<0.001	9.3	NS
2.0 mg/kg +24h	5/14	36	<0.001	10.2	NS
20 mg/kg toxicity	10/10	100	--	8.2	--
OLP-CDV					
20 mg/kg +24h	14/14	100	NS	8.1	NS
6.7 mg/kg +24h	12/14	86	NS	11.4	<0.01
2.0 mg/kg +24h	13/14	93	NS	12.4	0.001
20 mg/kg toxicity	10/10	100	--	9.7	--
OLE-CDV					
20 mg/kg +24h	14/14	100	NS	7.4	<0.001
6.7 mg/kg +24h	8/15	53	<0.01	13.0	NS
2.0 mg/kg +24h	8/15	53	<0.01	12.1	0.02
20 mg/kg toxicity	6/10	60	--	9.0	--

a. Compounds were prepared daily in water and delivered orally in 0.2 ml doses except CDV which was prepared in sterile saline and delivered ip. in 0.1 ml doses. Animals were treated once daily for 5 days beginning +24 hours post viral inoculation.
b. MDD = Mean Day of Death.
c. NS = Not significant when compared to the placebo control.

Effect of Oral Treatment with HDP-CDV, ODE-CDV, OLE-CDV, or OLP-CDV on the Mortality of BALB/c Mice Inoculated Intranasally with Cowpox, Brighton

	Mortality				
Treatment[a]	Number	Percent	P-value	MDD[b]	P-value
Placebo					
Saline +48h	15/15	100	---	9.5	---
CDV					
20 mg/kg +48h	1/15	7	<0.001	13.0	0.09
6.7 mg/kg +48h	0/15	0	<0.001	---	<0.001
2.0 mg/kg +48h	11/15	73	NS	13.5	<0.001
Placebo					
Water +48h	15/15	100	---	8.6	---
HDP-CDV					
20 mg/kg +48h	14/14	100	NS	9.9	<0.01
6.7 mg/kg +48h	12/14	86	NS	10.5	NS
2.0 mg/kg +48h	15/15	100	NS	9.7	0.02
ODE-CDV					
20 mg/kg +48h	15/15	100	NS	8.3	NS
6.7 mg/kg +48h	6/14	43	<0.01	12.7	0.01
2.0 mg/kg +48h	3/15	20	<0.001	15.0	NS
OLP-CDV					
20 mg/kg +48h	14/14	100	NS	10.4	<0.001
6.7 mg/kg +48h	4/14	29	<0.001	12.5	0.09
2.0 mg/kg +48h	15/15	100	NS	11.9	<0.001
OLE-CDV					
20 mg/kg +48h	14/14	100	NS	9.5	0.02
6.7 mg/kg +48h	5/15	33	<0.001	12.0	<0.001
2.0 mg/kg +48h	12/15	80	NS	11.0	<0.001

a. Compounds were prepared daily in water and delivered orally in 0.2 ml doses except CDV which was prepared in sterile saline and delivered ip. in 0.1 ml doses. Animals were treated once daily for 5 days beginning +48 hours post viral inoculation.
b. MDD = Mean Day of Death.
c. NS = Not significant when compared to the placebo control.

Effect of Oral Treatment with HDP-CDV, ODE-CDV, OLE-CDV, or OLP-CDV on the Mortality of BALB/c Mice Inoculated Intranasally with Cowpox, Brighton

	Mortality				
Treatment[a]	Number	Percent	P-value	MDD[b]	P-value
Placebo					
Saline +72h	15/15	100	---	9.3	---
CDV					
20 mg/kg +72h	1/15	7	<0.001	20.0	0.08
6.7 mg/kg +72h	5/15	33	<0.001	13.2	<0.01
2.0 mg/kg +72h	15/15	100	NS	10.3	0.01
Placebo					
Water +72h	15/15	100	---	8.8	---
HDP-CDV					
20 mg/kg +72h	14/14	100	NS	12.8	<0.001
6.7 mg/kg +72h	7/15	47	<0.01	12.7	<0.001
2.0 mg/kg +72h	15/15	100	NS	10.7	<0.001
ODE-CDV					
20 mg/kg +72h	15/15	100	NS	10.4	<0.001
6.7 mg/kg +72h	7/13	54	0.02	11.6	0.07
2.0 mg/kg +72h	12/14	86	NS	9.8	0.02
OLP-CDV					
20 mg/kg +72h	15/15	100	NS	11.0	0.05
6.7 mg/kg +72h	12/14	86	NS	10.3	0.02
2.0 mg/kg +72h	15/15	100	NS	9.5	0.06
OLE-CDV					
20 mg/kg +72h	14/14	100	NS	10.4	0.001
6.7 mg/kg +72h	11/14	79	NS	11.5	0.02
2.0 mg/kg +72h	15/15	100	NS	10.1	0.01

a. Compounds were prepared daily in water and delivered orally in 0.2 ml doses except CDV which was prepared in sterile saline and was delivered ip. in 0.1 ml doses. Animals were treated once daily for 5 days beginning +72 hours post viral inoculation.
b. MDD = Mean Day of Death.
c. NS = Not significant when compared to the placebo control.

Effect of Oral Treatment with HDP-CDV (ARB#00-394) on the Mortality of BALB/c Mice Inoculated Intranasally with Vaccinia, WR

Treatment	Mortality		P-value	MDD	P-value
	Number	Percent			
Placebo					
Water +24h	15/15	100	---	6.8	---
HDP-CDV					
30 mg/kg +24h	14/15	93	NS	8.2	<0.01
10 mg/kg +24h	15/15	100	NS	9.7	<0.001
3 mg/kg +24h	4/15	27	<0.001	8.3	<0.01
1 mg/kg +24h	10/15	67	0.02	7.2	NS
Placebo					
Sterile saline +24h	15/15	100	---	7.7	---
CDV					
30 mg/kg +24h	0/15	0	<0.001	---	<0.001
10 mg/kg +24h	0/15	0	<0.001	---	<0.001
3 mg/kg +24h	0/15	0	<0.001	---	<0.001
1 mg/kg +24h	0/15	0	<0.001	---	<0.001
Placebo					
Water +48h	15/15	100	---	7.5	---
HDP-CDV					
30 mg/kg +48h	15/15	100	NS	9.7	<0.01
10 mg/kg +48h	15/15	100	NS	11.8	<0.001
3 mg/kg +48h	12/15	80	NS	8.3	NS
1 mg/kg +48h	14/15	93	NS	7.3	NS
Placebo					
Sterile saline +48h	15/15	100	---	7.8	---
CDV					
30 mg/kg +48h	1/15	7	<0.001	8.0	NS
10 mg/kg +48h	1/15	7	<0.001	13.0	0.06
3 mg/kg +48h	0/15	0	<0.001	---	<0.001
1 mg/kg +48h	4/15	27	<0.001	8.0	NS
HDP-CDV Toxicity					
30 mg/kg +24h	10/10	100	---	8.5	---
10 mg/kg +24h	1/10	10	---	6.0	---

HDP-CDV was prepared in water and delivered orally in 0.2 ml doses. CDV was prepared in sterile saline and was delivered ip. in 0.1 ml doses. Animals were treated once daily for 5 days beginning +24 and +48h post viral inoculation.
MDD = Mean Day of Death.
NS = Not significant when compared to the placebo control.

Toxicity of Oral Treatment with HDP-CDV, ODE-CDV, OLE-CDV or OLP-CDV in 3 week old of BALB/c Mice

	Mortality		
Treatment[a]	Number	Percent	MDD[b]
HDP-CDV			
20 mg/kg	6/10	60	9.8
15 mg/kg	6/10	60	6.3
10 mg/kg	0/10	0	---
5 mg/kg	0/10	0	---
ODE-CDV			
20 mg/kg	10/10	100	8.5
15 mg/kg	10/10	100	9.8
10 mg/kg	2/9	22	13.0
5 mg/kg	2/10	20	7.5
OLP-CDV			
20 mg/kg	10/10	100	9.2
15 mg/kg	7/10	70	9.9
10 mg/kg	2/10	20	8.5
5 mg/kg	0/10	0	---
OLE-CDV			
20 mg/kg	10/10	100	10.4
15 mg/kg	7/10	70	10.4
10 mg/kg	1/10	10	11.0
5 mg/kg	0/10	0	---

ARB compounds were prepared daily in water and delivered orally in 0.2 ml doses.
Animals were treated once daily for 7 days.
MDD = Mean Day of Death.

A4. Animal Results: R. Mark Buller, St. Louis University; Lethal Ectromelia Virus Challenge Results with Oral CDV analogs

Data provided by Dr. R. Mark Buller

Ectromelia virus aerosol challenge of A/J mice treated with 30 mg/kg of cidofovir and alkoxyalkyl esters of cidofovir for five days

Drug[1]	Drug ID#	Morbidity on day 7 pi[2]	DOD	Mean DOD ± STDEV	Mortality at day 16 pi
CDV	CDV	11-++	9,9,10	9.3±0.5	9/11[3] (82%)
HDP-CDV	KA116-46A	11-+	NA[4]	NA	0/11(0%)
ODE-CDV	KA116-46B	6-+++ 5-++++	9	NA	10/11[3] (91%)
OLP-CDV	KA116-46C	11-+	NA	NA	0/11 (0%)
OLE-CDV	KA116-46D	11-++	9	NA	1/11 (9%)
Control	(dH_2O)	1-++++ 3-++	7,7,7,7,7,7,7, 8,8,8,	7.3±0.5	11/11[3] (100%)

[1] Mice were treated daily with 30mg/kg drug by gavage on days 0 through 4. Infection by aerosol occurred ~ 4 hrs **prior** to the first dose of drug. The presented aerosol dose was 6.4×10^4 PFU.

[2] Morbidity=0, healthy, no signs of sickness; +, face fur ruffled, no conjunctivitis; ++, face and body fur ruffled, hunched posture, eyes starting to look swollen; +++, face and body fur ruffled, hunched posture, both eyes have conjuctivitis; ++++, near death-same as above, plus eyes swollen shut, little or no movement, marked respiratory distress (mice with this level of sickness are euthanized).

[3] Mice euthanized due to morbidity: CDV 6/11; ODE-CDV 9/11; and control 1/11.

[4] Not applicable.

Ectromelia virus aerosol challenge of A/J mice treated with 10 mg/kg of cidofovir and alkoxyalkyl

Drug[1]	Drug ID#	Morbidity on day 7 pi[2]	DOD	Mean DOD ± STDEV	Mortality at day 16 pi
CDV	CDV	8-+++ 3-++++	8,8,8,8,8,9,9	8.3±0.5	11/11[3] (100%)
HDP-CDV	KA116-46A	11-+	15,15	15±0	2/11(18%)
ODE-CDV	KA116-46B	11-+	15	NA[4]	1/11 (9%)
OLP-CDV	KA116-46C	11-++	16	NA	1/11 (9%)
OLE-CDV	KA116-46D	5-+ 6-++	15	NA	1/11 (9%)
Control	(dH_2O)	6-+++ 1-++++	7,7,7,7,8,8,8,8	7.5±0.5	11/11[3] (100%)

esters of cidofovir for five days

[1] Mice were treated daily with 10mg/kg of drug by gavage on days 0 through 4. Infection by aerosol occurred ~4 hrs **prior** to the first drug. The presented aerosol dose was 2.6×10^6 PFU.

[2] Morbidity=0, healthy, no signs of sickness; +, face fur ruffled, no conjunctivitis; ++, face and body fur ruffled, hunched posture, eyes starting to look swollen; +++, face and body fur ruffled, hunched posture, both eyes have conjuctivitis; ++++, near death-same as above, plus eyes swollen shut, little or no movement, marked respiratory distress (mice with this level of sickness are euthanized).

[3] Mortality includes euthanasia due to morbidity: CDV 4/11; control 3/11.

[4] Not applicable.

A5. Animal Results: Don Smee, State University of Utah; Lethal Cowpox and Vaccinia Virus Data with Oral CDV Analogs

Data of Dr. Don Smee, State University of Utah

Expt. NIA-282. Effects of once-daily oral (p.o.) treatments with HDP-cidofovir compared to intraperitoneal (i.p.) or p.o. treatments with cidofovir on a lethal cowpox virus respiratory infection in mice.

		Uninfected Tox. Controls		Infected, Treated		
Compound (mg/kg/day)	Treatment Route	Survivors/ Total	Mean Host Wt. Change[a] (g)	Survivors/ Total	Mean Day of Death[b]	Mean lung Virus Titer[c]
HDP-cidofovir (3)	p.o.	5/5	+0.2	1/10	11.4±2.5**	8.1±0.2
HDP-cidofovir (1)	p.o.	5/5	+0.9	1/10	10.1±1.8*	8.1±0.1
HDP-cidofovir (0.3)	p.o.	5/5	+0.7	0/10	8.8±0.6	8.1±0.2
HDP-cidofovir (0.1)	p.o.	5/5	+0.5	0/10	8.8±0.9	8.2±0.2
Cidofovir (30)	p.o.	5/5	+0.1	0/10	10.8±1.8**	8.1±0.2
Cidofovir (30)	i.p.	5/5	-0.1	8/9***	14.0±1.8***	7.4±0.2***
Placebo	p.o.	5/5	+0.7	0/10	8.6±0.7	8.3±0.1

[a] Difference between starting (day 0) weight and day 6 weight.

[b] Of mice that died prior to day 21.

[c] Log_{10} PFU/g, determined from mice sacrificed on day 5 of the infection.

* P<0.05, **P<0.01, ***P,0.001.

Animals: Female 13-15 g BALB/c mice
Virus: Cowpox (Brighton strain)
Drug Diluent: Water (oral)
Saline (i.p.)

Treatment Schedule: Once daily for 5 days starting 24 hours after virus exposure
Treatment route: Oral (by gavage) or i.p.
Experiment duration: 21 Days

Expt. NIA-284. Effects of single oral (p.o.) treatments with HDP-cidofovir compared to intraperitoneal (i.p.) treatment with cidofovir on a lethal cowpox virus respiratory infection in mice.

		Uninfected Tox. Controls		Infected, Treated		
Compound (mg/kg)	Treatment Route	Survivors/ Total	Mean Host Wt. Change[a] (g)	Survivors/ Total	Mean Day of Death[b]	Mean lung Virus Titer[c]
HDP-cidofovir (100)	p.o.	5/5	-1.2	7/10**	11.3±0.6**	7.2±0.4***
HDP-cidofovir (30)	p.o.	5/5	-0.6	1/10	13.3±2.1***	8.1±0.2*
DHP-cidofovir (10)	p.o.	5/5	+0.6	1/10	10.7±3.0*	8.2±0.2
Cidofovir (100)	p.o.	5/5	0.0	6/10*	9.3±1.5	7.8±0.4**
Placebo	p.o.	5/5	+0.8	0/10	8.6±0.5	8.4±0.1

[a] Difference between starting (day 0) weight and day 2 weight.

[b] Of mice that died prior to day 21.

[c] Log_{10} PFU/g, determined from mice sacrificed on day 5 of the infection.

* P<0.05, **P<0.01, ***P,0.001.

Animals: Female 13-15 g BALB/c mice
Virus: Cowpox (Brighton strain)
Drug Diluent: Water (oral)
Saline (i.p.)

Treatment Schedule: One time only given
24 hours after virus exposure
Treatment route: p.o. (by gavage) or i.p.
Experiment duration: 21 Days

Expt. NIA-295. Effects of single treatments with HDP-cidofovir or cidofovir on a lethal vaccinia virus respiratory infection in mice

Compound (mg/kg)	Treatment Route	Survivors/ Total	Mean Day Of Death[a]	Mean Lung Parameters (day 5) Score[b]	Weight (mg)	Virus Titer[c]
HDP-cidofovir (100)	oral	0/10	8.9 ± 1.4	0.0 ± 0.0	140 ± 25***	8.5 ± 0.2**
HDP-cidofovir (30)	oral	0/10	10.2 ± 0.6***	0.1 ± 0.2	164 ± 26**	8.7 ± 0.2*
HDP-cidofovir (10)	oral	0/10	8.4 ± 1.3	0.0 ± 0.0	220 ± 29	8.8 ± 0.1
Cidofovir (100)	i.p.	8/10***	7.5 ± 2.1	0.0 ± 0.0	158 ± 17***	8.3 ± 0.2***
Placebo	oral	0/10	8.1 ± 1.2	0.0 ± 0.0	245 ± 24	9.0 ± 0.1

[a] Of mice that died prior to day 21.

[b] Lung consolidation score of 0 (normal) to 4 (total lung discoloration).

[c] Log_{10} PFU/g..

* P<0.05, **P<0.01, ***P,0.001.

Animals: Female 13-15 g BALB/c mice
Virus: Vaccinia (WR strain)
Drug Diluent: Sterile saline
Treatment Schedule: One time only given 24 hours after virus exposure
Treatment route: Oral or intraperitoneal i.p.
Experiment duration: 21 Day

A6. Published Manuscript:

Kern, E.R., C. Hartline, C., Harden, E., Keith, K., Rodriguez, N, Beadle, J.R. and Hostetler, K.Y., Enhanced inhibition of orthopoxvirus replication *in vitro* by alkoxyalkyl esters of cidofovir and cyclic cidofovir, Antimicrobial Agents Chemotherapy, 46:991-995 , 2002.

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Alkoxyalkyl Esters of Cidofovir and Cyclic Cidofovir Exhibit Multiple-Log Enhancement of Antiviral Activity against Cytomegalovirus and Herpesvirus Replication In Vitro

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The incidence of cytomegalovirus (CMV) retinitis is declining in AIDS patients but remains a significant clinical problem in patients with organ transplants and bone marrow transplants. Prophylaxis with ganciclovir (GCV) or valganciclovir reduces the incidence of CMV disease but may lead to the emergence of drug-resistant virus with mutations in the UL97 or UL54 gene. It would be useful to have other types of oral therapy for CMV disease. We synthesized hexadecyloxypropyl and octadecyloxyethyl derivatives of cyclic cidofovir (cCDV) and cidofovir (CDV) and found that these novel analogs had 2.5- to 4-log increases in antiviral activity against CMV compared to the activities of unmodified CDV and cCDV. Multiple-log increases in activity were noted against laboratory CMV strains and various CMV clinical isolates including GCV-resistant strains with mutations in the UL97 and UL54 genes. Preliminary cell studies suggest that the increase in antiviral activity may be partially explained by a much greater cell penetration of the novel analogs. 1-*O*-Hexadecyloxypropyl-CDV, 1-*O*-octadecyloxyethyl-CDV, and their corresponding cCDV analogs are worthy of further preclinical evaluation for treatment and prevention of CMV and herpes simplex virus infections in humans.

Although the incidence and prevalence of cytomegalovirus (CMV) retinitis in AIDS patients are declining due to the use of highly active antiretroviral therapies (12), CMV continues to be a major cause of opportunistic infections in patients with allogeneic bone marrow transplants (BMTs) and solid-organ transplants (6). In transplant patients, the incidence of CMV infection increases with the duration and degree of immunosuppression, approximating 70% in allogeneic BMT patients who are CMV seropositive (2) and in patients receiving solid-organ transplants from CMV-seropositive donors (4, 18). CMV disease is associated with a high risk of morbidity and mortality in solid-organ transplant and allogeneic BMT patients (6). While prophylaxis with ganciclovir (GCV) significantly reduces the incidence of CMV disease in transplant recipients, drug resistance may emerge because of mutations in the UL97 gene, which catalyzes the initial phosphorylation of GCV, or in the UL54 polymerase gene of the virus (for a review, see reference 5). Current therapies for CMV disease in transplant patients are based primarily on intravenous therapy with GCV, cidofovir (CDV), or foscarnet (phosphonoformate) or, more recently, with oral valganciclovir.

It would be useful to identify more effective oral therapies for the treatment of CMV disease in allogeneic bone marrow, stem cell, or solid-organ transplant patients and in CMV retinitis patients with AIDS. We have developed a strategy to improve the antiviral activity and oral absorption of acyclovir (ACV) and GCV by covalently attaching alkoxyalkyl or alkoxyglyceryl residues to the phosphate of ACV monophosphate or GCV monophosphate (1, 8, 9). These ether lipid analogs generally show severalfold increases in activity over the activity of underivatized ACV or GCV and provide increased oral absorption in rodents (8). In woodchucks with hepatitis, 1-*O*-hexadecyloxypropyl-phospho-ACV reduced woodchuck hepatitis virus DNA levels in plasma by nearly 2 logs after 4 weeks of treatment with 10 mg/kg of body weight twice daily, but a five times greater oral dose of ACV (molar basis) had no effect (7).

To determine if more effective and less toxic forms of CDV or cyclic CDV (cCDV) can be designed, we synthesized several alkoxyalkyl analogs of these compounds and evaluated their antiviral activities against human CMV (HCMV) and herpes simplex virus (HSV) by DNA reduction and plaque reduction assays with cells infected with various wild-type and GCV-resistant strains of CMV and HSV type 1 (HSV-1). Surprisingly, we detected multiple-log enhancement of the in vitro antiviral activities of the alkoxyalkyl analogs compared with the activity of underivatized cCDV or CDV.

MATERIALS AND METHODS

Chemistry. (i) General. All products were homogeneous by thin-layer chromatography (TLC), performed on Analtech 250-μm Silica Gel GF Uniplates and visualized under UV light with phospray (Supelco, Bellafonte, Pa.) and by charring. Chromatographic purification was done by the flash method with Merck silica gel 60 (240 to 400 mesh). ^{1}H and ^{31}P nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz on a Varian HG-400 spectrophotometer with tetramethylsilane (internal) and 85% D_3PO_4 in D_2O (external) as references for ^{1}H and ^{31}P (0.00 ppm), respectively. Electrospray ionization mass spectroscopy (ESI) was performed by Mass Consortium (San Diego, Calif.). CDV (compound 1) was provided by Gilead Sciences, Inc. (Foster City, Calif.). The synthesis and

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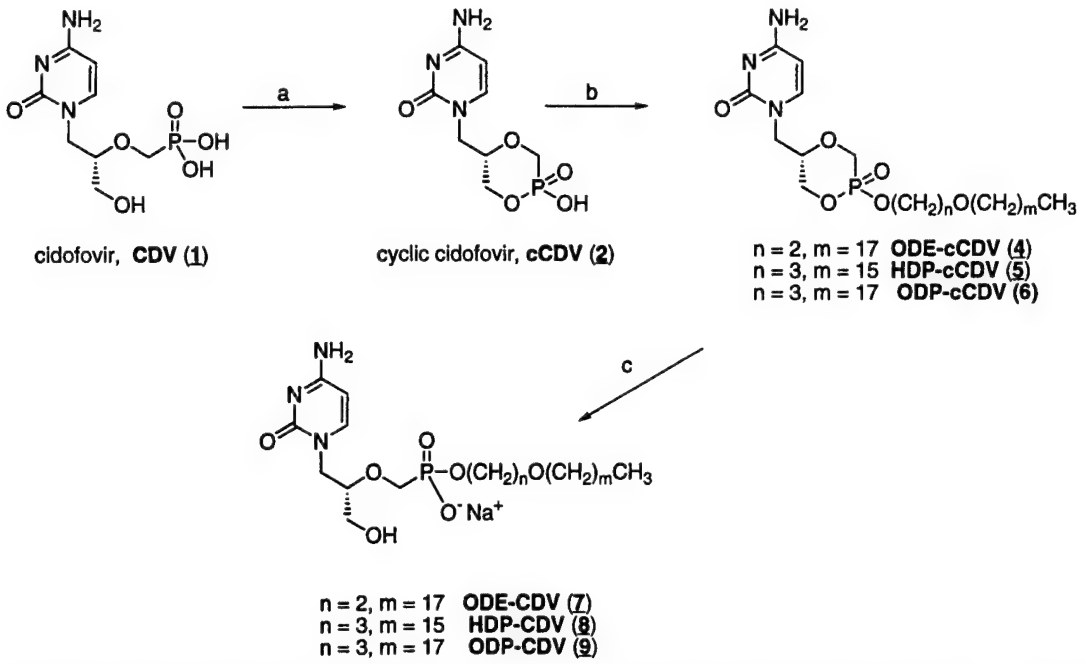

FIG. 1. Synthesis of alkoxyalkyl analogs of CDV and cCDV. Reagents: a, *N,N*-dicyclohexyl-morpholinocarboxamidine, 1,3-dicyclohexylcarbodiimide, pyridine, 100°C; b, ODE-Br, ODP-Br, or HDP-Br, *N,N*-dimethylformamide, 80°C; c, 0.5 M NaOH. The underscored numbers in parentheses correspond to the compound numbers used throughout the report.

characterization of compounds 2, 4, 5, 7, and 8 have been reported previously (10) (Fig. 1).

1-Bromo-3-octadecyloxypropane. Triphenylphosphine (10.0 g, 38 mmol) was added to a cooled (0°C) solution of 3-octadecyloxy-1-propanol (5.0 g, 15 mmol) and carbon tetrabromide (10.6 g, 32 mmol) in dichloromethane (70 ml) in 2-g portions over 30 min. The reaction mixture was stirred for 45 min at 0°C and then for 1 h at room temperature. The reaction mixture was concentrated, and the residue was dissolved in ether. After the mixture was stirred for 1 h, it was filtered and the filtrate was concentrated. The residue was purified by flash chromatography. Elution with 90% hexane–10% ethyl acetate yielded 4.3 g (77%) as a colorless oil. ^{1}H NMR δ 0.88 (t, 3-H), 1.25 (br s, 30-H), 1.56 (m, 2-H), 2.09 (p, 2-H), 3.42 (t, 2-H), 3.49 to 3.54 (two triplets, 4-H). Electrospray mass spectroscopy (MS), positive and negative, failed to give a molecular ion.

(ii) cCDV-hexadecyl ester (compound 3). A mixture of compound 2 (188 mg, 0.34 mmol) and 1-bromohexadecane (520 mg, 1.8 mmol) in *N,N*-dimethylformamide (25 ml) was stirred and heated to 80°C for 6 h. The mixture was concentrated, and the residue was purified by flash chromatography. Elution with 10% methanol (MeOH)–90% CH_2Cl_2 yielded 58 mg of compound 3 (33%) as a white powder. ^{1}H NMR δ (dimethyl sulfoxide [DMSO]-d_6) 0.85 (t, 3-H), 1.23 (broad s, 26-H), 1.60 (m, 2-H), 3.55 to 4.20 (m, 9-H), 5.6 (dd, 1-H), 7.18 and 7.04 (broad d, 2-H), 7.57 and 7.45 (d, 1-H); ^{31}P NMR δ +13.60 and +12.48 (mixture of axial and equatorial diastereomers) (13); MS (ESI) *m/z* 486 $(M^+ H)^+$, 484 $(M^- H)^-$. TLC R_f = 0.9 ($CHCl_3$-MeOH-concentrated NH_4OH-H_2O; 80:20:1:1).

(iii) cCDV-octadecyloxypropyl ester (compound 6). A mixture of compound 2 (1.02 g, 1.8 mmol) and 1-bromo-3-octadecyloxypropane (2.82 g, 7.5 mmol) in *N,N*-dimethylformamide (35 ml) was stirred and heated (80°C) for 6 h. The mixture was then concentrated in vacuo, and the residue was purified by flash chromatography. Elution with 10% MeOH–90% CH_2Cl_2 afforded 450 mg of a white powder (41% yield). High-pressure liquid chromatography, TLC, and spectroscopic analysis showed the presence of two diastereomeric (axial and equatorial) alkylation products. ^{1}H NMR δ (DMSO-d_6) 0.85 (t, 3-H), 1.23 (broad s, 30-H), 1.47 (m, 2-H), 1.84 (p, 2-H), 3.55 to 4.20 (m,13-H), 5.70 (dd, 1-H), 7.18 and 7.04 (broad d, 2-H), 7.55 and 7.45 (d, 1-H); ^{31}P NMR δ +13.61 and +12.31; MS (ESI) *m/z* 572 $(M^+ H)^+$, 570 $(M^- H)^-$. TLC R_f = 0.9 ($CHCl_3$-MeOH-concentrated NH_4OH-H_2O; 80:20:1:1).

(iv) CDV-octadecyloxypropyl ester (compound 9). Compound 6 (230 mg, 0.38 mmol) was dissolved in 0.5 M NaOH (5 ml), and the mixture was stirred at room temperature for 1.5 h. The solution was neutralized with acetic acid, and the precipitate was isolated by filtration and then purified by flash column chromatography. The product (133 mg, 58%) was eluted with CH_2Cl_2-MeOH (70:30). ^{1}H NMR δ (DMSO-d_6) 0.86 (t, 3-H), 1.24 (broad s, 30-H), 1.47 (m, 2-H), 1.73 (p, 2-H), 3.20 to 3.89 (m, 13-H), 5.72 (m, 1-H), 7.21 (d, 2-H), 7.54 (d, 1-H); ^{31}P NMR δ +13.98; MS (ESI) *m/z* 584 $(M^+ Na)^+$, 560 $(M^- H)^-$. TLC R_f = 0.27 ($CHCl_3$-MeOH-concentrated NH_4OH-H_2O; 80:20:1:1).

Preparation of control and drug-containing liposomes for antiviral assays. For the in vitro studies, 1-*O*-hexadecyloxypropyl-cCDV (HDP-cCDV), 1-*O*-hexadecyloxypropyl-CDV (HDP-CDV), 1-*O*-octadecyloxyethyl-cCDV (ODE-cCDV), 1-*O*-octadecyloxyethyl-CDV (ODE-CDV), 1-*O*-octadecyloxypropyl-cCDV (ODP-cCDV), 1-*O*-octadecyloxypropyl-CDV (ODP-CDV), and hexadecyl-cCDV (HD-cCDV) were incorporated into liposomes. Chloroform solutions of the phospholipids, cholesterol (CH), and drugs were mixed to provide dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), CH, and an alkoxyalkanol-CDV or alkoxyalkanol-cCDV analog at a molar ratio of 50/10/30/10. Control liposomes were prepared without drug and had a DOPC-DOPG-CH composition of 60/10/30. The chloroform was removed under a stream of nitrogen, and the thin lipid film was hydrated by the addition of 360 μl of 250 mM sorbitol–20 mM sodium acetate (pH 5.5). The small multiple-dose vial was sealed under nitrogen with a Teflon-lined cap and sonicated for 1 h at 42°C. The clear preparation of sonicated liposomes, representing a nominal drug concentration of 5 mM, was diluted sequentially with Dulbecco's modified Eagle's medium containing 4% fetal bovine serum to provide the indicated range of concentrations, and the medium was added to the virus-infected cells as indicated below.

Antiviral assays for activities against various strains of CMV and HSV-1 in vitro. Antiviral activity against HCMV (AD169) or HSV-1 was determined by a DNA reduction assay with MRC-5 human lung fibroblast cells with DNA probes supplied by Diagnostic Hybrids, Athens, Ohio, as described previously (9) or by plaque reduction assay with human foreskin fibroblast cells infected with various strains of HCMV or HSV-1 (11). The results of antiviral assays with HDP-CDV presented to cells in dilute DMSO were similar to those obtained with the compound presented to cells in liposomes, and blank liposome controls had no effect on viral replication. The antiviral activities of the various alkoxyalkyl esters of CDV and cCDV were also determined in CMV-infected murine, rat, and guinea pig embryonic fibroblast cells by plaque reduction assays (11, 14). The cytotoxic concentration of drug which reduced the viable cell number by 50% (CC_{50}) was determined. In the plaque reduction assays, cytotoxicity was determined by measurement of neutral red uptake (14).

TABLE 1. Antiviral activities and selectivities of CDV compounds in HCMV or HSV-1-infected MRC-5 human lung fibroblast measured by DNA reduction assay[a]

Compound	EC_{50} (μM)		Toxicity (CC_{50} [μM])	Selectivity for:	
	HCMV	HSV-1		HCMV	HSV-1
CDV	0.46 ± 0.08 (4)	3.3 ± 3.7 (3)	>1,000	>303	>2,174
ODE-CDV	$2 \times 10^{-5} \pm 3 \times 10^{-5}$ (6)	0.001 ± 0.002 (3)	210	2×10^5	10×10^6
HDP-CDV	$2 \times 10^{-6} \pm 3 \times 10^{-6}$ (4)	0.0001 ± 0.0001 (4)	10	1×10^5	5×10^6
ODP-CDV	$3 \times 10^{-5} \pm 4 \times 10^{-5}$ (4)	0.003 ± 0.001 (3)	32	1×10^4	1×10^6
cCDV	0.47 ± 0.13 (3)	2.3 ± 1.5 (3)	>1,000	>2,128	>435
HD-cCDV	0.04 ± 0.01 (3)	3.1 ± 2.4 (3)	6.5	163	2.0
ODE-cCDV	$1 \times 10^{-4} \pm 1 \times 10^{-4}$ (4)	0.005 ± 0.005 (3)	320	3×10^6	6×10^4
HDP-cCDV	$3 \times 10^{-4} \pm 3 \times 10^{-4}$ (6)	0.04 ± 0.03 (3)	320	1×10^6	8×10^3
ODP-cCDV	$3 \times 10^{-4} \pm 3 \times 10^{-4}$ (3)	0.35 ± 0.18 (3)	320	1×10^6	900

[a] The values are means ± standard deviations. Numbers in parentheses represent number of replicates. Selectivity is CC_{50}/EC_{50}.

RESULTS

MRC-5 human lung fibroblasts were infected with HCMV (AD169) or HSV-1, and the antiviral activities of CDV and cCDV were assessed by DNA reduction assay (Table 1). Against HCMV the 50% effective concentrations (EC_{50}s) for CDV and cCDV were similar (0.46 to 0.47 μM). The alkoxyalkyl analogs ODE-CDV, ODP-CDV, and HDP-CDV were 4 to 5 logs more active against HCMV, with EC_{50}s ranging from 2×10^{-6} to 3×10^{-5} μM. In cells infected with HSV-1, CDV and cCDV reduced viral replication by 50% at 3.3 and 2.3 μM, respectively. Again, the alkoxyalkyl analogs of CDV were most active, with EC_{50}s of 0.0001 to 0.003 μM. HDP-CDV was the most active of these three compounds. The alkoxyalkyl analogs of cCDV were less active than the corresponding CDV compounds. We also synthesized the 16-carbon straight-chain alkyl ester of cCDV, HD-cCDV, which lacks the oxygen group two or three carbons from the ester functionality. Interestingly, this compound is 133 to 400 times less active than ODE- or HDP-cCDV, esters of octadecylethanediol and hexadecylpropanediol, respectively (Table 1). The cytotoxicities of the alkoxyalkyl esters of CDV and cCDV in MRC-5 cells were greater than those observed with CDV or cCDV, but the selectivities of the HDP, ODE, and ODP derivatives of cCDV and CDV against CMV or HSV-1 increased greatly because of the marked increases in antiviral activity. In contrast, the compound lacking the oxygen heteroatom in the alkyl chain, HD-cCDV, exhibited greater toxicity and less antiviral activity (Table 1).

We also evaluated the activities of the analogs of CDV and cCDV against HSV-1 and HSV-2 by the plaque reduction assay. CDV and cCDV appeared to be less active against HSV-1 by the plaque reduction assay (Table 2) than by the DNA reduction assay, with EC_{50}s of 18.0 and 30.6 μM, respectively, compared with EC_{50}s of 3.3 and 2.3 μM, respectively, by the DNA reduction assay (Table 1). The EC_{50}s of the alkoxyalkyl analogs were also higher by the plaque reduction assay than by the DNA reduction assay. Nevertheless, increases in antiviral activity of 2.39 to 2.81 logs were noted with HDP-CDV and ODE-CDV, respectively, compared with the activity of unmodified CDV. Somewhat lesser increases in activity were noted with the analogs of cCDV versus those of unmodified cCDV when the activities were measured by the plaque reduction assay (Table 2).

The antiviral activities of the HDP and ODE analogs of cCDV and CDV were also examined by the plaque reduction assay with human foreskin fibroblast cells infected with various laboratory strains and clinical isolates of HCMV, and the antiviral activities of these compounds were compared with those of GCV, cCDV, and CDV (Table 3). In general, when the antiviral activities of CDV and cCDV were compared to those of the respective HDP and ODE esters, multiple-log increases in antiviral activities were observed. For example, for strain AD169, the EC_{50} of CDV was 0.38 μM, whereas the EC_{50}s of both HDP-CDV and ODE-CDV were 0.0009 μM, representing increases in activity of 2.6 logs for the new analogs. Similar results were obtained with the Towne, Davis, and C9208/5-4-2 strains of wild-type HCMV (Table 3). Although the Toledo strain was much less sensitive to CDV (EC_{50}, 13.8 μM), the HDP-CDV and ODE-CDV analogs were both substantially more active, with EC_{50}s of 0.025 μM, representing an increase in antiviral activity of 2.74 logs. Nearly 3-log increases in antiviral activities were noted with the alkoxyalkanol analogs of CDV against cells infected with the Coffman, C8708/17-1-1, and C9208/5-4-2 strains of CMV. Similar findings were obtained with HDP-cCDV and ODE-cCDV, except that these analogs were generally somewhat less active than the corresponding analogs of CDV (Table 3).

The activities of GCV, CDV, cCDV, and the alkoxyalkyl esters of CDV and cCDV were also evaluated against a panel of drug-resistant HCMV mutants kindly provided to E. R. Kern by Karen Biron of GlaxoSmithKline, Research Triangle Park, N.C., and Donald Coen, Boston, Mass. (Table 4). The EC_{50}s of GCV for GCV-resistant strains with mutations in the

TABLE 2. Activities of alkoxyalkyl esters of CDV and cCDV against HSV-1 and HSV-2 by plaque reduction assay

Compound	EC_{50} (μM)			
	HSV-1		HSV-2	
	Assay 1	Assay 2	Assay 1	Assay 2
CDV	22.5	7.9	13.2	7.9
HDP-CDV	0.08	0.04	0.13	0.03
ODE-CDV	0.03	0.012	0.03	0.03
cCDV	28.4	10.6	11.2	7.6
HDP-cCDV	0.9	0.25	0.35	0.11
ODP-cCDV	0.5	0.2	0.28	0.12

TABLE 3. Activities of GCV, CDV, cCDV, and alkoxyalkyl esters of CDV and cCDV against HCMV replication measured by plaque reduction assay

Isolate[a]	EC_{50} (μM)[b]						
	GCV	CDV	HDP-CDV	ODE-CDV	cCDV	HDP-cCDV	ODE-cCDV
AD169	2.75 ± 1.6	0.38 ± 0	0.0009 ± 0.0001	0.0009 ± 0.0001	0.31 ± 0.02	0.001 ± 0	0.0018 ± 0.001
Towne	4.3 ± 0	0.4 ± 0.11	0.0009 ± 0	0.0009 ± 0.0001	0.48 ± .02	0.001 ± 0	0.001 ± 0
Davis	5.1 ± 0	0.66 ± 0.3	0.00095 ± 0.00007	0.0009 ± 0.0001	0.45 ± 0.07	0.001 ± 0	0.001 ± 0
Toledo	19.6 ± 7.2	13.8 ± 7.3	0.025 ± 0.007	0.025 ± 0.02	17.1 ± 8	0.055 ± 0.03	0.055 ± 0.03
Coffman	4.7 ± 0	0.87 ± 0.15	0.001 ± 0	0.001 ± 0	1.2 ± 0.5	0.0015 ± 0.0007	0.002 ± 0.0007
C8708/17-1-1	2.6 ± 0.9	1.1 ± 0.5	0.001 ± 0	0.001 ± 0	2.1 ± 0.4	0.0015 ± 0.0007	0.0025 ± 0.002
C9208/3-3-1	4.25 ± 1.2	0.95 ± 0.6	0.001 ± 0	0.001 ± 0	1.3 ± 0.3	0.00095 ± 0.00007	0.001 ± 0
C9208/5-4-2	1.6 ± 0.42	0.41 ± 0.09	0.00085 ± 0.0002	0.001 ± 0	1.1 ± 0.26	0.00095 ± 0.00007	0.001 ± 0

[a] All isolates are wild type.
[b] The values are the means ± standard deviations of two or more determinations.

UL97 gene were 3.7 to 16.4 times greater than the average EC_{50} (3.61 μM) for the seven wild-type strains (Table 5). CDV and cCDV retained nearly full activity against the strains with mutations in the UL97 gene, and their alkoxyalkyl esters were 2.5 to 2.98 logs more active than the underivatized nucleotide phosphonates. A mutant of CMV with a mutation in the DNA polymerase gene (UL54), mutant GDGP53, exhibited 15 to 22 times greater resistance to cCDV and CDV and 15 times greater resistance to GCV than the wild type. Interestingly, the alkoxyalkyl esters of cCDV and CDV retained substantial activities against this mutant with a mutation in the DNA polymerase gene; HDP-CDV and ODE-CDV both had EC_{50}s of 0.02 μM, a 3.4-log increase in activity compared with that of unmodified CDV. HDP-cCDV and ODE-cCDV were also active, showing 2.4- to 2.5-log increases in activity compared with that of cCDV against the mutant with the polymerase mutation. A double mutant, mutant 759D100, which has mutations in both the DNA polymerase (G987A in UL54) and in UL97 (deletion of 590 to 593 in UL97) was the mutant most resistant to GCV, but it was somewhat less cross resistant than the mutant with a mutation in the polymerase gene, mutant GDGP53. The antiviral activities of the alkoxyalkanol analogs of cCDV and CDV against mutant 759D100 were intermediate between those against the mutant with the UL97 gene mutation and the mutant with the polymerase gene mutation. Against mutant 759D100, the HDP and ODE esters of cCDV and CDV were 2.2 to 2.5 logs more active than the unmodified phosphonates (Table 5).

HDP-CDV and ODE-CDV were also highly active against a panel of strains of CMV from animals, including CMV strains from the mouse, rat, and guinea pig. The most active compounds were HDP-CDV and ODE-CDV, with EC_{50}s of 0.0009 to 0.005 μM, whereas the EC_{50} of unmodified CDV was 0.26 μM. Similar trends were noted with the cCDV series of compounds (Table 6).

DISCUSSION

The covalent addition of an alkoxyalkyl ester group to the phosphonate of CDV or cCDV resulted in remarkable increases in antiviral activities against CMV and HSV-1 in vitro. The cytotoxicities of the analogs were also increased, but selectivity (CC_{50}/EC_{50}) was increased substantially in most cases. When the activities were measured by plaque reduction assay, the increases in activities observed with the alkoxyalkyl analogs of CDV and cCDV were 1 to 2 logs less than those noted by DNA reduction assay. However, the activities of CDV and cCDV were similar by the two assays. Alkyl ether analogs of both ethanediol and propanediol were highly active. Although we have not done extensive structure-activity analyses, both 16- and 18-carbon alkyl ether chains were highly effective. By the DNA reduction assay, the analogs of cCDV were less active than the corresponding CDV compounds. Interestingly, when cCDV was coupled directly to hexadecanol, a long-chain alcohol 16 carbons in length, a 2-log drop in antiviral activity was noted, demonstrating the importance of the oxygen heteroatom. The oxygen heteroatom may make the analogs subject to rapid enzymatic conversion to cCDV or CDV, precursors of the active antiviral CDV diphosphate (CDV-PP). This must be confirmed by metabolic studies comparing the conversion to cCDV by using radiolabeled HD-cCDV and HDP-cCDV incubated with cell homogenates or subcellular membrane fractions. Preliminary studies indicate that HDP-CDV is metabolized by an intracellular enzyme of the phospholipase C type (unpublished observation).

The HDP and ODE derivatives of CDV exhibited 2.5- to 4-log increases in antiviral activity depending on the antiviral assay used (Tables 1 and 2). The mechanism of the increased activity remains to be determined. However, CDV enters cells by pinocytosis, which may greatly restrict passage of unmodified drug into cells. Preliminary studies in our laboratory with ^{14}C-labeled CDV and HDP-CDV indicate that the amount of HDP-CDV that enters the cell is increased by several logs; intracellular CDV-PP can easily be detected when 10 μM HDP-CDV is used, but when 10 μM CDV is used, the intracellular levels of CDV-PP are substantially lower (unpublished data). Full assessment of the mechanisms of the increased

TABLE 4. Drug-resistant HCMV isolates

Isolate	Drug(s) to which the isolate is resistant	Affected gene	Mutation	Reference
C9209/1-4-4[a]	GCV	UL97	M460V	3
C8914-6[a]	GCV	UL97	L595F	3
C8805/37-1-1[a]	GCV	UL97	M460V	3, 15
GDGP53[b]	GCV, CDV	UL54 (Pol)	G987A	16
759D100[b]	GCV	UL54/UL97	G987A/Δ590–93	17

[a] Provided by Karen Biron.
[b] Provided by Donald Coen.

TABLE 5. Effects of GCV, CDV, cCDV, and alkoxyalkyl esters against drug-resistant isolates of HCMV by plaque reduction assay[a]

Isolate	EC_{50} (μM)						
	GCV	CDV	HDP-CDV	ODE-CDV	cCDV	HDP-cCDV	ODE-cCDV
Wild type[a]	3.61 ± 1.3	0.68 ± 0.29	0.0009 ± 0.0006	0.00096 ± 0.00005	0.99 ± 0.63	0.0011 ± 0.0003	0.0015 ± 0.0006
Drug-resistant mutants							
C9209/1-4-4	59.3 ± 0.2	2.3 ± 0.28	0.003 ± 0	0.0040 ± 0.001	1.95 ± 0.9	0.005 ± 0	0.0045 ± 0.0007
C8914-6	13.5 ± 2	0.99 ± 0.01	0.0025 ± 0.0007	0.0010 ± 0	1.45 ± 0.21	0.005 ± 0.001	0.0045 ± 0.0007
C8805/37-1-1	47.4 ± 1.1	0.84 ± 0.2	0.00095 ± 0.00007	0.00095 ± 0.00007	0.96 ± 0.2	0.001 ± 0	0.0010 ± 0
GDGP53	54.6 ± 23	15.7 ± 14.1	0.020 ± 0.009	0.020 ± 0.008	15.9 ± 12.2	0.06 ± 0.05	0.050 ± 0.03
759D100	177 ± 28.2	2.0 ± 0.56	0.0065 ± 0.0007	0.0055 ± 0.0007	2.5 ± 0.1	0.015 ± 0.007	0.0150 ± 0.004

[a] The values are the means ± standard deviations for the seven wild-type strains for which the results are presented in Table 3 (the Toledo strain was omitted from the analysis).

activities of HDP-CDV and ODE-CDV awaits determination of comparative levels of intracellular CDV monophosphate and CDV-PP in cells incubated with equimolar concentrations of ^{14}C-labeled CDV and the respective analogs. However, our preliminary studies suggest that enhanced cell uptake is a major factor in the 2.5- to 4-log increases in antiviral activity which have been documented.

The alkoxyalkyl analogs of CDV and cCDV also showed multiple-log increases in antiviral activities against multiple clinical isolates of HCMV (Table 3). One of these isolates, Towne, exhibited reduced susceptibility to GCV, cCDV, and CDV, with EC_{50}s 3 to 10 times greater than those for laboratory HCMV and clinical isolates. Although the Towne strain was also relatively resistant to the alkoxyalkyl analogs of CDV, the EC_{50}s were still low (0.025 to 0.055 μM) and were more than 2.5 logs lower than those of underivatized CDV or cCDV. In addition, all of the alkoxyalkyl analogs tested had multiple-log increases in activity compared with that of CDV against GCV-resistant HCMV mutants (Table 5). Most of these strains have mutations in the UL97 gene, which controls the phosphorylation of GCV. Interestingly, a DNA polymerase mutant, mutant GDGP53, which exhibits about 10-fold greater resistance to CDV and cCDV and 50-fold greater resistance to GCV than the wild type, remains sensitive to HDP-CDV and ODE-CDV, with EC_{50}s of 0.02 μM (Table 5). Although this represents a 20-fold decrease in antiviral activity compared with that of wild-type strains of HCMV, HDP-CDV may still be useful against mutants of this type in vivo. Similar results were observed with a double mutant with mutations in both the UL97 and the UL54 genes.

HDP-CDV and ODE-CDV are also highly active against orthopoxviruses such as vaccinia virus and cowpox virus (10) and monkeypox virus and smallpox virus (John Huggins, personal communication). The EC_{50}s of HDP-CDV and ODE-CDV for the various poxviruses are in the range of 0.01 to 0.8 μM, making these agents of interest as potential treatments for smallpox, should the disease reappear.

In conclusion, long-chain alkyl ethers of propanediol or ethanediol covalently linked to cCDV or CDV provide multiple-log increases in antiviral activity against laboratory wild-type strains, various clinical isolates, and GCV-resistant strains of HCMV in vitro. The most active compounds were HDP-CDV and ODE-CDV, with EC_{50}s of 2×10^{-6} and 2×10^{-5} μM, respectively. Based on our previous research, compounds of this type may be orally bioavailable (6–8). Further evaluation of this approach is warranted to assess the suitability of HDP-CDV and ODE-CDV for further development for the treatment or prevention of human infections with the herpesvirus group of viruses and poxviruses.

TABLE 6. Activities of alkoxyalkyl esters of CDV and cCDV against murine, rat, and guinea pig CMV strains

Compound	EC_{50} (μM)[a]		
	MCMV	RCMV	GpCMV
GCV	9.0 ± 4.9	48.2 ± 1.1	239.0 ± 10.6
CDV	0.26 ± 0.02	0.30 ± 0.03	0.31 ± 0.003
HDP-CDV	0.0009 ± 0	0.004 ± 0.001	0.0009 ± 0.00007
ODE-CDV	0.001 ± 0	0.005 ± 0	0.0009 ± 0.00007
cCDV	0.39 ± 0.05	0.46 ± 0.05	0.50 ± 0.26
HDP-cCDV	0.003 ± 0	0.005 ± 0	0.001 ± 0
ODE-cCDV	0.004 ± 0	0.005 ± 0	0.001 ± 0

[a] The values are the means ± standard deviations of two assays. Abbreviations: MCMV, murine CMV; RCMV, rat CMV; GpCMV, guinea pig CMV.

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Enhanced Inhibition of Orthopoxvirus Replication In Vitro by Alkoxyalkyl Esters of Cidofovir and Cyclic Cidofovir

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The nucleotide phosphonates cidofovir (CDV) and cyclic cidofovir (cCDV) are potent antiviral compounds when administered parenterally but are not well absorbed orally. These compounds have been reported to have activity against orthopoxvirus replication in vitro and in animal models when administered parenterally or by aerosol. To obtain better oral activity, we synthesized a novel series of analogs of CDV and cCDV by esterification with two long-chain alkoxyalkanols, 3-hexadecyloxy-1-propanol (HDP-CDV; HDP-cCDV) or 3-octadecyloxy-1-ethanol (ODE-CDV; ODE-cCDV). Their activities were evaluated and compared with those of CDV and cCDV in human foreskin fibroblast (HFF) cells infected with vaccinia virus (VV) or cowpox virus (CV) using a plaque reduction assay. The 50% effective concentrations (EC_{50}s) against VV in HFF cells for CDV and cCDV were 46.2 and 50.6 μM compared with 0.84 and 3.8 μM for HDP-CDV and HDP-cCDV, respectively. The EC_{50}s for ODE-CDV and ODE-cCDV were 0.20 and 1.1 μM, respectively. The HDP analogs were 57- and 13-fold more active than the parent nucleotides, whereas the ODE analogs were 231- and 46-fold more active than the unmodified CDV and cCDV. Similar results were obtained using CV. Cytotoxicity studies indicated that although the analogs were more toxic than the parent nucleotides, the selective index was increased by 4- to 13-fold. These results indicate that the alkoxyalkyl esters of CDV and cCDV have enhanced activity in vitro and need to be evaluated for their oral absorption and efficacy in animal models.

Since smallpox was considered to be eradicated in the 1970s, there has been little activity in developing antiviral agents for this infection (10). However, in view of the threat of bioterrorism using variola virus or other orthopoxviruses, such as monkeypox virus, which continues to infect humans in central Africa, there is a renewed need to develop antiviral agents for these viruses (3, 11, 12, 17, 18, 24). For many years the laboratory of Erik De Clercq and other laboratories have utilized in vitro and animal models with vaccinia virus (VV) to screen potential antiviral compounds for activity against poxviruses and have identified a few active agents. Methisazone, ribavirin, idoxuridine, interferon, interferon inducers, S2442, and cidofovir (CDV) have been identified as potential therapies for these infections (7, 8, 9, 20, 21, 23). Of particular interest was the finding that CDV and other phosphonate nucleotides were inhibitory to this group of viruses, including VV, cowpox virus (CV), camelpox virus, monkeypox virus, and variola virus (J. W. Huggins, personal communication). The activity of CDV is of particular interest as a potential therapy for smallpox, as it is already approved for the treatment of cytomegalovirus (CMV) infections and has been shown to have activity in animal models using VV and CV (2, 22, 26, 27). We have confirmed the activity of CDV against both VV and CV in tissue culture and animal models in our laboratory (4) and report here the results of some new alkoxyalkyl esters of CDV and cyclic CDV (cCDV). Although CDV and cCDV are potent inhibitors of orthopoxvirus replication in vitro and are highly effective in animal models when inoculated parenterally, they are absorbed poorly when given orally (1, 5).

Previous studies from our group have shown that alkylglycerol phosphate or alkoxypropyl phosphate esters of acyclovir (14) and ganciclovir (16) have greater oral bioavailability than the parent compounds in rodents. Furthermore, these compounds are active orally in animal models of herpesvirus disease (16) and hepatitis (15). To obtain better oral activity with CDV, we synthesized a new series of analogs by esterification using two long-chain alkoxyalkanols, 3-hexadecyloxy-1-propanol (HDP-CDV; HDP-cCDV) and 3-octadecyloxy-1-ethanol (ODE-CDV; ODE-cCDV) (Fig. 1). We then compared their activities with those of CDV and cCDV in human foreskin fibroblast (HFF) cells infected with strains of VV or CV using a plaque reduction assay. The cytotoxicities of the compounds were determined using a neutral-red uptake (50% cytotoxic concentration [CC_{50}]) or cell proliferation (50% inhibitory concentration [IC_{50}]) assay.

MATERIALS AND METHODS

Chemistry. (i) General. Thin-layer chromatography was performed on Analtech 250-μm-thick silica gel GF Uniplates and visualized by UV, phospray (Supelco, Bellafonte, Pa.), and charring. Chromatographic purification was done by the flash method using Merck silica gel 60, 240 to 400 mesh. ^1H and ^{31}P nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz on a Varian HG-400 spectrophotometer with tetramethylsilane (internal) or 85% D_3PO_4 in D_2O (external) as ^1H and ^{31}P references (0.00 ppm), respectively. Mass spectroscopy (MS) was performed by Mass Consortium (San Diego, Calif.). CDV (Vistide) (compound 1) was purchased from a retail pharmacy or was provided by Gilead Sciences, Inc. (Foster City, Calif.).

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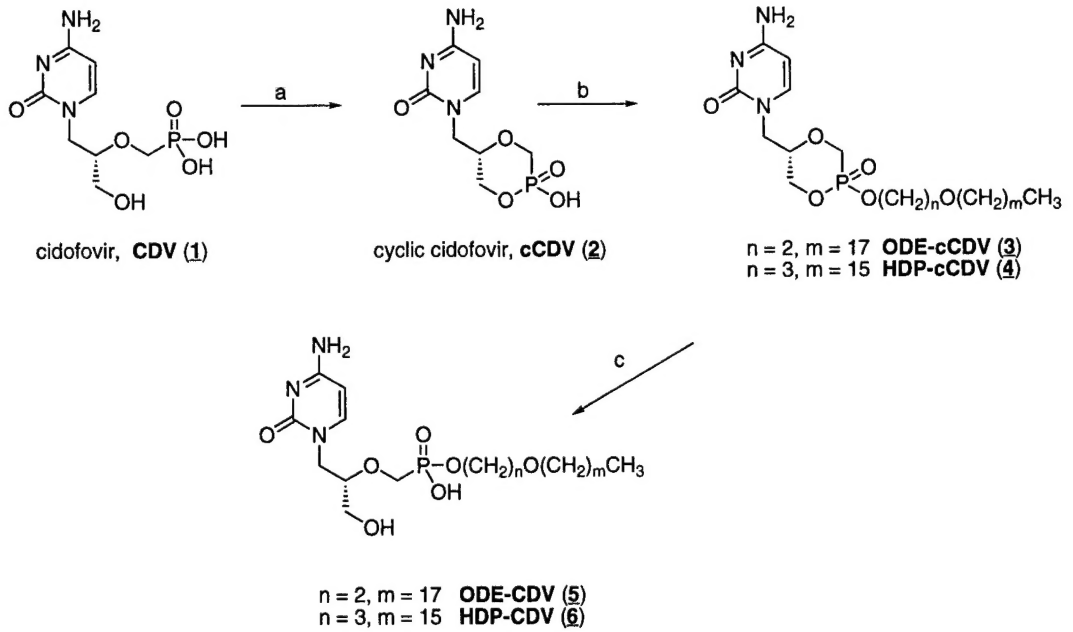

FIG. 1. Synthesis and structures of alkoxyalkyl analogs of CDV and cCDV. The arrows indicate the following reagents: (a) *N,N*-dicyclohexylmorpholinocarboxamide, *N,N*-dicyclohexylcarbodiimide, pyridine, 100°C; (b) 1-bromo-3-octadecyloxyethane (ODE), or 1-bromo-3-hexadecyloxypropane (HDP), *N,N*-dimethylformamide, 80°C; (c) 0.5 M NaOH. The numbers in parentheses are the compound numbers (see the text).

(ii) cCDV dicyclohexyl-morpholinocarboxamidine salt (compound 2). cCDV was prepared from CDV as described previously (1) except that the compound was isolated as the dicyclohexyl-morpholinocarboxamidine salt. Thus, to a stirred suspension of CDV (1.0 g; 3.17 mmol) in *N,N*-dimethylformamide (25 ml) was added *N,N*-dicyclohexyl-4-morpholinecarboxamidine (1.03 g; 3.5 mmol). The reaction mixture was stirred for 12 h at room temperature, during which the CDV dissolved. This solution was then added slowly to a hot pyridine (25 ml) solution of 1,3-dicyclohexyl carbodiimide (1.63 g; 7.9 mmol). The mixture was stirred at 100°C for 16 h, cooled to room temperature, and filtered, and the filtrate was concentrated under vacuum. The residue was purified by chromatography. Elution with 60% MeOH–40% CH_2Cl_2 gave compound 2 as a white solid.

(iii) 1-Bromo-3-octadecyloxyethane. To a cooled (0°C) solution of 3-octadecyloxy-1-ethanol (10 g; 31.8 mmol) and carbon tetrabromide (21.1 g; 64 mmol) was added triphenylphosphine (21.3 g; 81 mmol) in 4-g portions over 30 min. The reaction mixture was stirred for 45 min at 0°C and then for 1 h at room temperature. The reaction mixture was concentrated, and the residue was dissolved in ether. After being stirred for 1 h, the mixture was filtered, and the filtrate was concentrated. The residue was purified by flash chromatography. Elution with 90% hexane–10% ethyl acetate yielded 8.0 g (67%). ^{1}H NMR 0.88 (t, 3H), 1.25 (br s, 32H), 1.57 (m, 2H), 3.47 (q, 2H), 3.74 (t, 2H).

(iv) 1-Bromo-3-hexadecyloxypropane. To a cooled (0°C) solution of 3-hexadecyloxy-1-propanol (4.7g; 16 mmol) and carbon tetrabromide (11.2 g; 34 mmol) was added triphenylphosphine in 2-g portions over 30 min. The reaction was stirred for 45 min at 0°C and then for 1 h at room temperature. The brominated product was isolated as described above. ^{1}H NMR 0.88 (t, 3H), 1.25 (broad s, 28H), 1.56 (m, 2H), 2.09 (p, 2H), 3.43 (t, 2H), 3.52 (q, 2H).

(v) cCDV-octadecyloxyethyl ester (compound 3). A mixture of compound 2 (1.0 g; 1.8 mmol) and 1-bromo-3-octadecyloxyethane (3.0 g; 7.9 mmol) in *N,N*-dimethylformamide (35 ml) was stirred and heated to 80°C for 6 h. The mixture was concentrated, and the residue was purified by flash chromatography. Elution with 10% MeOH–90% CH_2Cl_2 yielded 320 mg (33%). ^{1}H NMR (dimethyl sulfoxide [DMSO]-d_6) 0.87 (t, 3H), 1.23 (broad s, 32H), 1.47 (m, 2H), 3.55 B 4.20 (m, 11H), 5.65 (dd, 1H), 7.18 and 7.04 (broad s, 1 H), 7.55 and 7.45 (d, 1H), 8.30 (broad s, 2H); ^{31}P NMR +13.88 and +12.62; MS electrospray ionization [EI]) *m/z* 558 $(M + H)^+$, 556 $(M - H)^-$.

(vi) cCDV-hexadecyloxypropyl ester (compound 4). A mixture of compound 2 (500 mg; 0.9 mmol) and 1-bromo-3-hexadecyloxypropane (1.8 g; 5 mmol) in *N,N*-dimethylformamide (35 ml) was stirred and heated (80°C) for 6 h. The mixture was then concentrated in vacuo, and the residue was purified by flash chromatography. Elution with 10% MeOH–CH_2Cl_2 afforded 150 mg of a white powder (31% yield). High-performance liquid chromatography, thin-layer chromatography, and spectroscopic analysis showed the presence of two diastereomeric (axial and equatorial) alkylation products. ^{1}H NMR (DMSO-d_6) 0.85 (t, 3H), 1.23 (broad s, 28H), 1.47 (m, 2H), 1.84 (p, 2H), 3.55 B 4.20 (m, 11H), 5.65 (dd, 1H), 7.18 and 7.04 (broad s, 1 H), 7.55 and 7.45 (d, 1H), 8.30 (broad s, 2H); ^{31}P NMR +13.88 and +12.62; MS (EI) *m/z* 544 $(M + H)^+$, 542 $(M - H)^-$.

(vii) CDV-octadecyloxyethyl ester (compound 5). Compound 3 (160 mg; 0.3 mmol) was dissolved in 0.5 M NaOH (20 ml) and stirred at room temperature for 1.5 h. The solution was neutralized with acetic acid. The precipitate was collected by filtration. ^{31}P NMR +13.98; MS (EI) *m/z* 598 $(M + Na)^+$, 574 $(M - H)^-$.

(viii) CDV-hexadecyloxypropyl ester (compound 6). Compound 4 (130 mg; 0.23 mmol) was dissolved in 0.5 M NaOH (5 ml) and stirred at room temperature for 1.5 h. The solution was neutralized with acetic acid, and the precipitate was isolated by filtration and then purified by flash column chromatography. The product (105 mg) was eluted with 70:30 CH_2Cl_2–MeOH. ^{1}H NMR (DMSO-d_6) 0.86 (t, 3H), 1.24 (broad s, 28H), 1.47 (m, 2H), 1.73 (p, 2H), 3.20 B 3.89 (m, 11H), 5.72 (m, 1H), 7.21 (d, 1 H), 7.54 (d, 1H), 8.23 (broad s, 2H); ^{31}P NMR +13.98; MS (EI) *m/z* 584 $(M + Na)^+$, 560 $(M - H)^-$.

Virus pool preparation. The VV strain Copenhagen and CV strain Brighton stock pools were obtained from John Huggins of the U.S. Army Medical Research Institute for Infectious Diseases, Frederick, Md. These pools had been prepared in Vero cells and were diluted 1:50 in our laboratory to provide working stocks. VV strains WR, Elstree, IHD, and NYC were obtained from the American Type Culture Collection and propagated in HFF cells.

Plaque reduction assay for efficacy. Two days prior to use, HFF cells were plated on six-well plates and incubated at 37°C with 10% CO_2 and 90% humidity. On the day of assay, the drugs were made up at twice the desired concentration in 2× minimal essential medium (MEM) containing 5% fetal bovine serum (FBS) and antibiotics and diluted serially 1:5 in 2× MEM to provide six concentrations of drug. The initial starting concentration was usually 200 μM and ranged down to 0.06 μM. The virus to be used was diluted in MEM containing 10% FBS to a desired concentration which would give 20 to 30 plaques per well. The medium was then aspirated from the wells, and 0.2 ml of virus was added to each well in triplicate, with 0.2 ml of medium being added to drug toxicity wells. The plates were incubated for 1 h with shaking every 15 min. After the incubation period, an equal amount of 1% agarose was added to an equal volume of each drug dilution. This gave final drug concentrations beginning with 100 μM and ending with 0.03 μM and a final agarose overlay concentration of 0.5%. The drug-agarose mixture was added to each well in 2-ml volumes, and the plates

were incubated for 3 days, after which the cells were stained with a 1.5% solution of neutral red. After a 5- to 6-h incubation period, the stain was aspirated and the plaques were counted using a stereomicroscope at ×10 magnification. The MacSynergy II, version 1, computer program was used to calculate the 50% effective concentration (EC_{50}).

Neutral-red uptake assay for toxicity. Twenty-four hours prior to the assay, HFF cells were plated on 96-well plates at a concentration of 2.5×10^5 per ml. After 24 h, the medium was aspirated and 125 μl of drug was added to the first row of wells and then diluted serially 1:5 using the Beckman BioMek liquid-handling system. After the addition of the drug, the plates were incubated for 7 days in a CO_2 incubator at 37°C. At that time, the medium with drug was aspirated, and 200 μl of 0.01% neutral red in phosphate-buffered saline (PBS)/well was added and incubated for 1 h. The dye was aspirated, and the cells were washed with PBS using a Nunc plate washer. After the PBS was removed, 200 μl of 50% ethanol–1% glacial acetic acid (in H_2O)/well was added. The plates were placed on a rotary shaker for 15 min, and the optical densities were read at 540 nm on a Bio-tek plate reader. The CC_{50} was calculated using the program described previously.

Cell proliferation assay for toxicity. Twenty-four hours prior to the assay, HFF cells were seeded in 6-well plates at a concentration of 2.5×10^4 per well in MEM containing 10% FBS. On the day of the assay, the drugs were diluted serially in MEM containing 10% FBS at increments of 1:5 covering a range from 100 to 0.03 μM. The medium from the wells was aspirated, and 2 ml of each drug concentration was then added to each well. The cells were incubated in a CO_2 incubator at 37°C for 72 h. The medium-drug solution was removed, and 1 ml of 0.25% trypsin was added to each well and incubated until the cells started to come off the plate. The cell-trypsin mixture was pipetted up and down vigorously to provide a homogenous cell suspension, 0.2 ml of the mixture was added to 9.8 ml of Isoton III, and the cells were counted using a Coulter Counter. Each sample was counted three times with two replicate wells per sample. The IC_{50} was also calculated as described above.

RESULTS

The potential for use of a poxvirus as a bioterrorism agent or for the accidental spread of an indigenous pathogen, such as monkeypox virus, has resulted in a renewed effort in the development of both new antiviral therapies and new vaccines for prophylactic or therapeutic use. As part of an initial screening effort to identify an antiviral agent that could be rapidly developed for the treatment of poxvirus infections, we evaluated the activities of most of the currently approved antiviral drugs against both VV and CV infections in vitro. Since our laboratory uses HFF cells in our antiviral screening assays and most prior work has been carried out in Vero cells, we first compared the activities of a number of nucleosides and nonnucleosides that are approved for treatment of other viral infections in both HFF and Vero cells. The activities of these compounds against VV and CV are summarized in Table 1. The only compounds that exhibited significant activity in these assays were CDV, cCDV, fialuridine, and idoxuridine. In addition, the protease inhibitors nevirapine, nelfinavir, indinavir, saquinavir, and ritonavir were evaluated and had no activity. Since fialuridine and idoxuridine are both considered to be too toxic for parenteral administration to humans, CDV, which is currently approved for use in CMV infections in the immunocompromised host, appears to be the best choice for development for use against a potential poxvirus outbreak. Although CDV also has significant side effects, it may be acceptable for short-term use.

Based on our results with CDV, as well as those in the literature (9), we next evaluated other phosphonate nucleotides that are under evaluation for other diseases, such as AIDS and hepatitis. The results of these evaluations indicated that CDV, cCDV, adefovir dipivoxil, and 3-hydroxy-2-phosphonylmethoxy propyl adenine all had activity against VV and CV (data not presented). In essentially all cases, the compounds were as active, if not more so, in HFF cells as in Vero cells, so all subsequent assays were performed using HFF cells.

TABLE 1. Efficacies of antiviral agents against VV and CV in HFF and Vero cells

Drug	EC_{50} (μM)			
	VV		CV	
	HFF	Vero	HFF	Vero
Acyclovir	>144	>144	>444	>444
Ganciclovir	>392	NT[b]	NT	NT
CDV	23 ± 4.1[a]	30 ± 12.6[a]	48 ± 1.8[a]	45 ± 7.9[a]
cCDV	21 ± 4.9[a]	90 ± 46[a]	51 ± 4.2[a]	132 ± 9.6[a]
Ribavirin	281	NT	>410	>246
Fialuridine	1.5 ± 0.05[a]	NT	0.24 ± 0.08[a]	NT
Zidovudine	>374	>374	>374	>374
Lamivudine	>436	>436	>436	>436
Didanosine	>423	>423	>423	>423
Zalcitibine	>474	>474	>474	>474
Stavudine	>446	>446	>446	>446
Idoxuridine	6.0 ± 0.2[a]	NT	2.0 ± 0.20[a]	NT

[a] Values are the means of two assays ± standard deviations.
[b] NT, not tested.

In the next experiment, we compared the activities of the two parent nucleotides with the newly synthesized alkoxyalkyl analogs, and the results are presented in Table 2. Both CDV and cCDV required about 40 to 50 μM to inhibit the replication of either VV or CV by 50%. In contrast, the alkoxyalkyl analogs, HDP-CDV and HDP-cCDV, were active at about 0.8 to 4.0 μM, and the ODE-CDV and ODE-cCDV analogs were active at 0.2 to 1.0 μM, respectively. The new analogs of CDV were 58- to 231-fold more active than the unmodified CDV against VV and 75- to 150-fold more active than CDV against CV. The alkoxyalkyl adducts of cCDV were about 13- to 97-fold more active against these viruses than the parent cCDV.

To guard against the possibility that the Copenhagen strain is not representative of VV strains, we determined the activities of the six phosphonate compounds against five strains of VV, and the results are summarized in Table 3. All six of the nucleotides were similarly active against the five strains of VV; however, the IHD and NYC strains appeared to be more susceptible than the Copenhagen, WR, or Elstree strain. Since there is only a single strain of CV, similar comparisons could not be made. The EC_{50}s for HDP-CDV ranged from 0.20 to 1.2 μM, while ODE-CDV EC_{50}s were 0.10 to 0.40 μM, rep-

TABLE 2. Activities of phosphonate nucleotides and alkoxyalkyl analogs against VV and CV in HFF cells

Compound	EC_{50} (μM)[a]	
	VV Copenhagen	CV Brighton
CDV	46.2 ± 11.9	44.7 ± 6.3
HDP-CDV	0.8 ± 0.4	0.6 ± 0.3
ODE-CDV	0.2 ± 0.1	0.3 ± 0.3
cCDV	50.6 ± 13.1	48.3 ± 8.0
HDP-cCDV	3.8 ± 1.5	2.1 ± 1.9
ODE-cCDV	1.1 ± 1.0	0.5 ± 0.1

[a] Values are the means of two assays ± standard deviations.

TABLE 3. Activities of phosphonate nucleotides and alkoxyalkyl analogs against strains of VV

Compound	EC_{50} (μM)[a]				
	Copenhagen	WR	Elstree	IHD	NYC
CDV	46.2 ± 11.9	45.8 ± 16.6	41.6 ± 22.4	13.4 ± 5.6	10.1 ± 1.3
HDP-CDV	0.8 ± 0.4	1.1 ± 1.0	1.2 ± 0.8	0.2 ± 0.0	0.4 ± 0.0
ODE-CDV	0.2 ± 0.1	0.24 ± 0.2	0.4 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
cCDV	50.6 ± 13.1	58.8 ± 10.9	41.6 ± 19.5	10.9 ± 1.3	10.3 ± 1.3
HDP-cCDV	3.8 ± 1.5	5.6 ± 1.3	3.8 ± 2.7	0.1 ± 0.0	0.1 ± 0.0
ODE-cCDV	1.1 ± 1.0	0.58 ± 0.2	0.5 ± 0.4	0.04 ± 0.02	0.03 ± 0

[a] Values are the means of two assays ± standard deviations.

TABLE 5. SI values of phosphonate nucleotides and alkoxyalkyl analogs in HFF cells

Compound	VV			CV		
	EC_{50} (μM)	CC_{50} (μM)	SI[a]	EC_{50} (μM)	CC_{50} (μM)	SI[a]
CDV	46.2	278	6	44.7	278	6.2
HDP-CDV	0.8	31	37	0.6	31	50
ODE-CDV	0.2	14.3	65	0.3	14.3	49
cCDV	50.6	>302	>6	48.3	>302	>6.2
HDP-cCDV	3.8	>100	>26	2.1	>100	>48
ODE-cCDV	1.1	47.8	43.4	0.5	47.8	88.5

[a] SI = CC_{50}/EC_{50}; EC_{50}s are from Table 2; CC_{50}s are from Table 4.

resenting 28- to 209-fold increases in activity versus CDV for these same strains. The HDP- and ODE-cCDV analogs were generally slightly less active, with EC_{50}s ranging from 0.10 to 5.6 and 0.03 to 1.1 μM, respectively, which represent increases of 11- to 343-fold over the activity of cCDV.

Since the evaluation of the toxicity of a new compound is an integral part of any drug development scheme, we next determined the cellular cytotoxicities of these compounds and their propensities for inhibition of cell proliferation. The cellular cytotoxicity (CC_{50}) and cell proliferation (IC_{50}) values for these compounds are listed in Table 4. In these assays, CDV and cCDV had relatively similar toxicities; however, the HDP analogs were about three- to ninefold more toxic than CDV or cCDV. The ODE analogs were 4- to 19-fold more toxic than either CDV or cCDV. The activity of a new compound, taking into account both its efficacy and toxicity, is often expressed as a selective index (SI), that is, the CC_{50} divided by the EC_{50}. The SI values for the six phosphonate nucleotide compounds are summarized in Table 5. The SI values for both CDV and cCDV were about 6, whereas those for the HDP analogs of CDV and cCDV were about 30. The SI values for the ODE analogs were 40 to 65, indicating that the analogs, although more toxic than the parent compounds, were more efficacious, which resulted in higher SI values.

DISCUSSION

There is at present no approved antiviral therapy that could be rapidly deployed in the event of a bioterrorism attack using a poxvirus or the unexpected spread of an indigenous agent like monkeypox virus to other parts of the world. Initial efforts in drug development for poxvirus infections have focused on the identification of compounds that are already approved for use against some other disease. Historically, a few compounds, including methisazone, ribavirin, idoxuridine, and fialuridine, have been reported to have various degrees of activity against a surrogate virus, VV (9). Our results also confirmed the activities of idoxuridine and fialuridine against both VV and CV; however, ribavirin was only slightly active in our assays. For a variety of reasons, including lack of clear efficacy, toxicity, or availability, none of these compounds are good possibilities for development for use against poxvirus infections.

Investigators from the laboratory of Erik DeClercq (8, 9, 20) first reported that a group of compounds often referred to as phosphonate nucleotides had activity against VV. These results have been confirmed by other laboratories, and the activity has been extended to other poxviruses, including cowpox virus (2, 4, 26), camel pox virus, monkey pox virus, and variola virus, the causative agent of smallpox (Huggins, personal communication). The best-known member of this class of compounds is CDV, which is currently approved for treatment of CMV infections in the immunocompromised host. This compound is a potent inhibitor of poxvirus replication in vitro (9) and has been shown to be very effective against both VV and CV infections in animal models, including in immunocompromised mice (2, 4, 22, 26, 27). Although there are other phosphonate nucleotides and their analogs, such as adefovir and adefovir dipivoxil, that are currently under evaluation in clinical studies for treatment of human immunodeficiency virus and/or hepatitis virus infections, there is far less information regarding their activity against poxvirus infections than is available for CDV (20).

Although CDV is approved for intravenous use in serious CMV infections in AIDS patients, there are a number of problems associated with its use. Two major problems that limit its use are its nephrotoxicity (13, 25) and its poor oral bioavailability. The toxicity of CDV may not be a limiting factor for its use during a poxvirus outbreak, as treatment would be expected to be of short duration. Additionally, since the drug has a long intracellular half-life, dosing can be infrequent, i.e., once or twice per week (2, 6, 19). Its lack of oral activity, however, would present much greater logistical problems, as the drug would need to be administered intravenously. A number of analogs of the phosphonate nucleotides, including cCDV and adefovir dipivoxil, have been synthesized to increase the oral bioavailability of these compounds. Another approach to improving the oral bioavailability of nucleosides has been to add certain ether lipid groups to increase oral absorption and cell membrane penetration. Members of our group have had pre-

TABLE 4. Cellular cytotoxicities (CC_{50}) and inhibition of cell proliferation (IC_{50}) of phosphonate nucleotides and alkoxyalkyl analogs in HFF cells

Compound	CC_{50} (μM)[a] (neutral-red uptake)	IC_{50} (μM)[a] (cell proliferation)
CDV	278.4 ± 9.2	19.5 ± 8.7
HDP-CDV	31 ± 2.1	1.7 ± 1.8
ODE-CDV	14.3 ± 9.7	0.5 ± 0.3
cCDV	>302 ± 0	27.8
HDP-cCDV	>100 ± 0	11.7 ± 4.5
ODE-cCDV	47.8 ± 57.3	2.2 ± 0.5

[a] Values are the means of two assays ± standard deviations.

vious experience utilizing this technology for improving the activity of nucleosides such as acyclovir, ganciclovir, penciclovir, and azidothymidine (14–16).

Earlier studies indicated that 1-0-hexadecylpropanediol-*P*-ganciclovir provided greater oral bioavailability in mice than ganciclovir and provided good oral activity against herpes simplex virus type 1 and murine CMV infections in mice (16). In addition, it was demonstrated that oral 1-0-hexadecyl-propanediol-*P*-acyclovir at 20 mg/kg of body weight daily lowered woodchuck hepatitis virus DNA levels by nearly 2 log units after 4 weeks of administration. Acyclovir at a fivefold-higher molar dose was not effective (15). To determine if a similar strategy could be employed with phosphonate nucleotides, we synthesized the 1-0-hexadecylpropanediol and the 1-0-octadecylethanediol esters of CDV and cCDV. As demonstrated in these studies, the new analogs were not only active against VV and CV replication in vitro, they were considerably more active than the parent compounds. Although there are no published reports of the efficacy of CDV for experimental variola virus infections, it was recently determined that CDV and cCDV inhibited the replication of variola virus strain Bangladesh in vitro at levels of about 25 μM. Importantly, the alkoxyalkyl analogs of CDV and cCDV were >100-fold more active than the unmodified compounds (John Huggins, unpublished results). The mechanism of action through which these analogs exhibit greater antiviral activity and selectivity is currently not well understood. However, preliminary studies using radiolabeled HDP-[^{14}C]CDV, have indicated that cellular uptake of the drug is many-fold greater than that observed with [^{14}C] CDV in human lung fibroblast cells (unpublished observations).

In summary, we have synthesized several alkoxyalkyl esters of CDV and cCDV which exhibited greatly enhanced antiviral activity and selectivity against two members of the orthopoxvirus group in tissue culture cells. Based upon our previous experiments with analogs of this type, we anticipate that these new compounds will exhibit significant oral activity compared with the parent compounds, and they now need to be evaluated in animal model systems for oral pharmacokinetics, toxicity, and antiviral efficacy against members of the orthopoxviruses.

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